A KINETIC STUDY OF SOLID PHASE PEPTIDE SYNTHESIS FOR THE PRODUCTION OF POLYPHENYLALANINE AND POLYSERINE WITH LOW EXCESS OF THE SYMMETRICAL ANHYDRIDE

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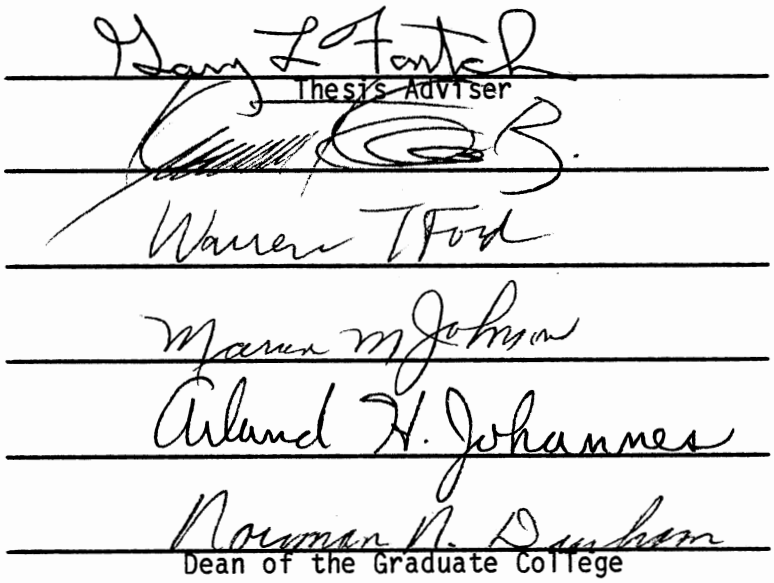

PREFACE

A continuous monitoring method for kinetic study of solid phase peptide synthesis was developed. This is a convenient way of measuring the rates of reactions in solvent/resin systems. The concentration of reagent change in the liquid phase, was followed by measuring ultraviolet absorbance. The resulting absorbance curves were analyzed to give the desired kinetic information.

An empirical kinetic model was proposed, and rate constants were determined over a range of reaction conditions (i.e., temperature, mixing rate, chain length, excess mole ratio, resin, etc.) for the synthesis of polyphenylalanine and polyserine. In general, the coupling rate between amino acids in solid phase peptide synthesis of polyamino acid decreased as peptide chain length increased, and increased as temperature increased; the higher excess mole ratio of carboxyl groups elevates the coupling rate, and when the number of amino acid residues increased, the effect extended. In addition, polystyrene crosslinked with one percent divinylbenzene showed faster coupling rates and less deviation from ideal second order rate kinetics than two percent crosslinking for this low excess mole ratio study.

For this heterogeneous reaction, film resistance to mass transfer was assumed not to be the rate determining step as a result of mixing rate experiments. Approximate values of activation energy based on two different reaction temperatures imply that the coupling reaction

corresponds more nearly with a diffusion-limited process. A simple reaction model, i.e., progressive-conversion, could not fully explain the phenomena observed in this study. Secondary structure of the peptide chain may have introduced an orientation problem of collision between amino acids and the amino terminus of the peptide-resin; this hypothesis was used to explain the experimental results.

Error analysis on the outside reactor volume, the circulation loop, was done by discrete approximation. Finally, recommendations are proposed for future study.

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NOMENCLATURE AND ABBREVIATIONS

Ⓑ	**blocking group**
Boc	tert-butyloxycarbonyl
Bpoc	2-(4-biphenylyl)propyl(2)oxycarbonyl
Bzl	benzyl
C_A	concentration of amino terminus (mole/lDCM)
C_X	concentration of symmetrical anhydride (mole/lDCM)
DCC	dicyclohexylcarbodiimide
DCM	dichloromethane
DCU	dicyclohexylurea
Ddz	2-(3,5-dimethoxyphenyl)propyl(2)oxycarbonyl
DMF	dimethylformamide
DVB	1,4-divinylbenzene
Fmoc	9-fluorenylmethyloxycarbonyl
Gly	glycine
His	histidine
Ile	isoleucine
k_1	pseudo-first order reaction rate constant, 1/sec
k_2	second order reaction rate constant, l/mol.sec
k_2'	apparent second order reaction constant, l/mol.sec
K_1	reaction rate constant of proposed rate expression, mmol/ml.sec
K_2	reaction rate constant of proposed rate expression, mmol/ml
N-	amino
Nps	2-nitrophenylsulfenyl

Pam	phenylacetamidomethyl
Phe	phenylalanine
Poc	2-phenylpropyl(2)oxycarbonyl
Ⓟ	protecting group
γ_A	rate of reaction, mmol/sec
Ⓡ	resin support
Ser	serine
SPPS	solid phase peptide synthesis
TEA	triethylamine
TFA	trifluoroacetic acid
Tmz	α,2,4,5,-tetramethylbenzyloxycarbonyl
Tos	4-toluenesulfonyl (tosyl)
UV	ultraviolet (spectroscopy)

CHAPTER I

INTRODUCTION

The solid phase peptide synthesis (SPPS) method was conceived by R.B. Merrifield in 1959 in an effort to overcome many of the problems associated with the solution methods for peptide synthesis. By 1962, Merrifield had established the commonly called "classical method" by demonstrating the formation of a tetrapeptide (1) and by synthesizing the 9-residue hormone, bradykinin (2). The major feature of SPPS is that the peptide is synthesized in a stepwise manner while it is attached by an ester bond to an insoluble solid support. The biggest advantage of this method is the absence of intermediate isolation steps thereby accelerating and simplifying synthesis of the peptide. SPPS bears an interesting similarity to the biological process of protein synthesis in which carboxyl activated amino acids are added to the N-terminus of a growing peptide chain which is bound to a solid support (the polyribosome).

The classical SPPS method contains several distinctive characteristics. First, the resin, solid support, used is chloromethylated polystyrene crosslinked with 1-2% divinylbenzene. The chloromethylated sites provide the anchoring base, and the low crosslinking percentage allows adequate swelling of the resin. The second feature of the classical method is that tert-butyloxycarbonyl (t-Boc or Boc) is used to protect the amine functionality while that amino acid is being added to

the peptide. The final characteristic involves the method utilized to couple the amino acids to form the peptide bonds. Dicyclohexylcarbodiimide (DCC) is either used directly, or the amino acid symmetrical anhydride, made separately by reaction with DCC, is used in the classical method. Although variations and modifications of every facet of Merrifield's method have been attempted, it still remains the most widely employed, and will be used in this study.

Because solid phase peptide synthesis is a repetitive addition of amino acids, a major concern with the method has been the heterogeneity of the final product which arises because the various reaction steps in the synthesis fail to reach completion. Successive partially incomplete additions result in both a low product yield and a mixture of similar peptides which are difficult to separate. To avoid these problems, many investigators allow the coupling step to continue substantially beyond the required time. Although this solution is acceptable on a lab-scale synthesis, a more efficient method is desirable for large scale production. Thus, information concerning the reaction dynamics must be, therefore, considered valuable for industrial use.

Knowledge of chemistry of SPPS is substantially advanced compared with the understanding of the kinetics for the reactions. The purpose of this study is to provide kinetic information of coupling reactions for use in SPPS reactor modeling and design. This was accomplished with a SPPS reactor connected to a UV-visible spectrophotometer. Kinetic data and reaction rate constants can be obtained using amino acids or amino acid derivatives which have useful ultraviolet spectra. As the coupling reaction progresses, amino acid is attached to the anchored peptide and the solution concentration decreases. This drop is detected

and recorded as the solution circulates through a flow cell in a U.V. spectrophotometer. The resulting absorbance curve can then be used as an aid for more efficient peptide production.

Four basic objectives will be addressed in this study. First, the experimental design will be refined. Next, repeated experiments of synthesizing homopolyphenylalanine and homopolyserine will be conducted to test both the success of the reactions and the accuracy of this monitoring method. Although polyamino acids have no biological activity, these biopolymers are being used as simple models in structure-property correlation studies of natural polypetides and protein. Then, a list of variables which might influence reaction kinetics and can be studied by this experimental set-up will be tested and qualitatively analyzed. These variables include peptide chain length, reaction temperature, mixing rate, mole ratio between the incoming amino acid derivative and the active site on the polymer resin and the degree of crosslinking of the polymer support. These results will be compared with results from the literature. Finally, the kinetic data obtained will be used to establish a kinetic model. The model rate constants will be determined.

These results should give new insight into the kinetics of SPPS, allow the study of biologically active peptides, and be used to improve design of industrial scale reactors.

CHAPTER II

LITERATURE REVIEW

Solid Phase Peptide Chemistry

The classical method of solid phase peptide synthesis basically is repetition of three chemical reactions, as illustrated in Figure 1. The rinsed resin or peptide resin is first subjected to treatment with a 1:3 solution of tribluoroacetic acid (TFA): dichloromethane (DCM). This removes the Boc protecting group on the terminal amine. This deprotection step is followed by treatment with 10% triethylamine (TEA) in DCM. TEA neutralizes the terminal end. The final step is acylation of the terminal amine by coupling of the desired amyl residue. Six solvent rinses with either DCM or dimethylformamide (DMF) are needed between each of the above steps. The details are described in EXPERIMENTAL PROCEDURE.

Several modified solid phase procedures have also been used and studied. The chemistry background of these modifications can be discussed in three aspects, namely; polymer support, peptide-resin link, and deprotection of α-amino groups.

A suitable insoluble support and a satisfactory means of attaching the first amino acid are of critical importance for successful SPPS. The standard 1% or 2% divinylbenzene cross-linked polystyrene, which is commonly used in polymer support reactions, were chosen by Merrifield and are currently the most popular polymer supports for SPPS research.

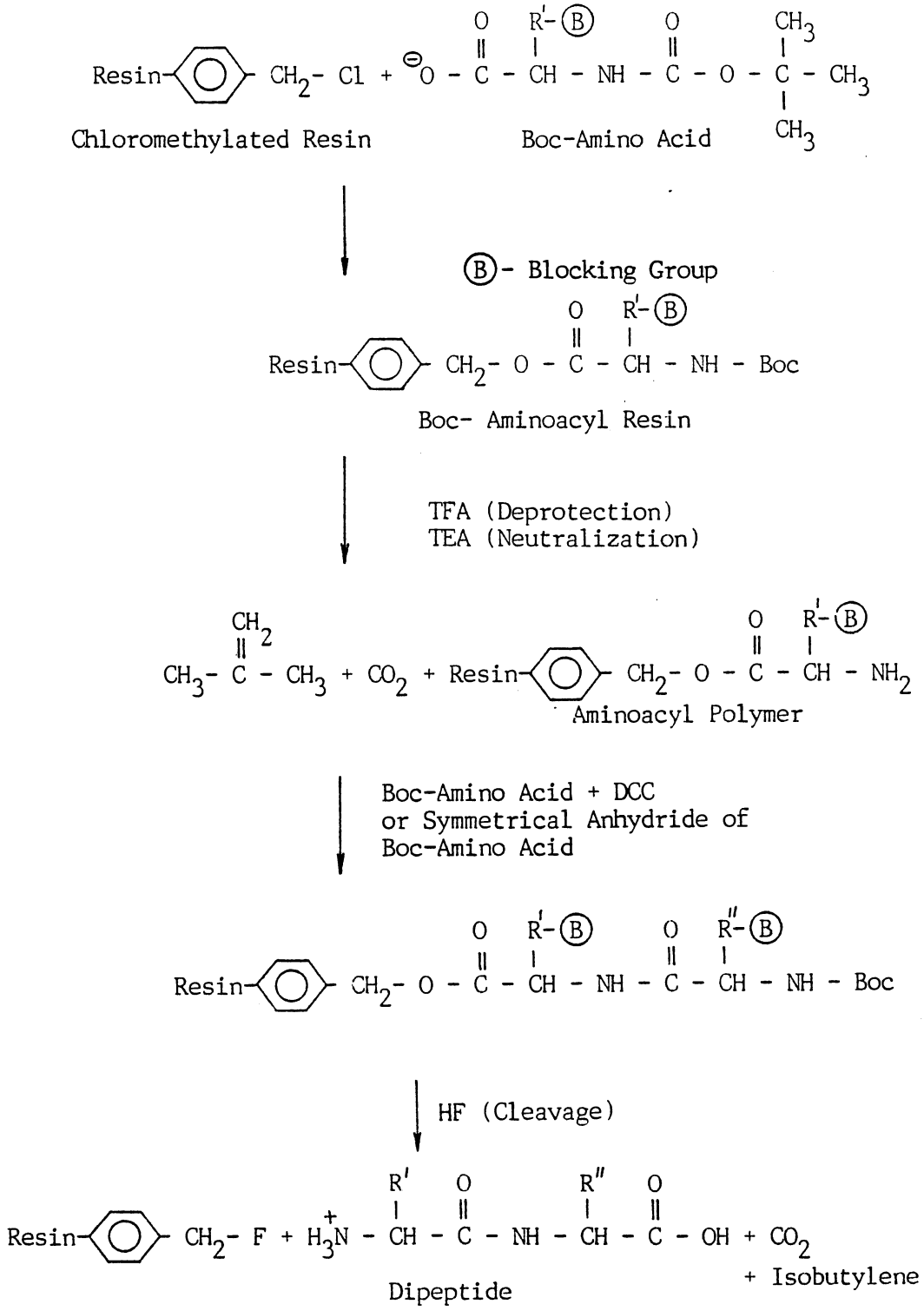


Figure 1. The Classical Merrifield Scheme of Solid Phase Peptide Synthesis.

But for some peptide sequences, solvents commonly used for SPPS, such as DCM, swell the resin effectively but would not be expected to solvate peptide chains very well. In this case, the incompatibility between the natures of the growing peptide chain and the polystyrene resin cause a significant percentage of growing peptide termination (3). In attempting to overcome these difficulties, Sheppard (4) developed a cross-linked polyamide resin, whose fundamental nature is very similar to that of peptide chains. Using the current SPPS technology, Sheppard and his coworkers showed that this polyamide resin made possible the synthesis of certain very difficult sequences, for instance, the C-terminal region of acyl carrier protein, ACP(65-74). In addition, polyamide supported on porous kieselguhr also opens new possibilities for continuous-flow synthesis systems (5).

In the classical SPPS system, the ester linking the peptide to the resin is only slightly labile to the reagents normally used for removal of Boc groups at each step of the synthesis. This might give a loss of about 1% per deprotection step by 25% TFA in DCM. This is not acceptable for synthesis of long peptides. A more stable peptide-resin link is needed and this can be achieved by substitution of the resin electron withdrawing groups. Pam resin is one of the modifications which increases the stability by a factor of 100 over that of the classical system. This stability appears to make Pam resin the choice for long peptide synthesis with Boc-amino acids. On the other hand, as peptides become larger and more complex, the peptides become less stable toward harsh deprotection or cleavage reagents such as HF and TFA. For this reason, labile peptide-resin links or extremely labile α-protecting groups are desired. In response to this challenge, Wang (6) developed

p-alkoxylbenzyl alcohol resin and coupling with Bpoc α-blocking groups. In this resin, the peptide-resin link is labilized by the electron-donating ability of ether oxygen in the para position of the ring.

The choice of protecting group depends not only on the nature of the group to be protected but also on the nature of other reactants to be used later in the synthesis. Protecting groups are chosen for their chemical stability which is much lower compared to the peptide bond. The most popular α-protecting group during the last two decades has been the Boc group. This group gives satisfactory lability-stability characteristics toward deprotection and cleavage reagents. As mentioned above, Bpoc is too labile for common use but is advantageous for long peptide synthesis. The difference in reactivity to acidolysis by TFA between Bpoc and Boc is approximately 120,000. Other protecting groups of intermediate reactivity have been designed and are labile enough for a more dilute TFA deprotection; these dilute TFA solutions should not harm the peptide-resin link in the classical system. Three such groups are the Ddz group of Birr (7), the Poc groups of Ragnarsson (8) and the Tmz group proposed by Matsuida and Stewart (9).

The choice for blocking groups of side chain functionalities is also subject to the stability-lability characteristic to deprotection and cleavage reagent. The blocking groups for individual amino acids are discussed in Stewart and Young (10).

Besides these aspects, several coupling reaction techniques attempting either to reduce reaction time or to improve coupling efficiency have also been tried. Basically, these techniques are devoted to improving activation of the carboxyl group by replacing -OH with a

better leaving group. The use of anhydrides, activated esters of amino acids (11), and various reagent additions to the coupling solution are examples of such improvements.

Large Scale Peptide Synthesis

A large scale SPPS method of producing molar quantities of peptide for clinical use has certain advantages over other synthetic process, like solution phase method, enzymatic synthesis; these advantages include a reactor which can be highly automated, one reactor which can produce many different peptides and with relatively few purification steps. However, a large scale SPPS operation must meet several criteria in order to produce acceptable product. Batch homogeneity and complete conversion are the most critical standards for a solid phase peptide reactor, and a good reproducibility with respect to yield and chemical properties are also important for this batchwise process.

A middle-size SPPS reactor has been reported to produce the 9-residue peptide, Serum Thymic Factor (FTS), in good yield and reproducibility (12). The coupling reactions have been carried out by mixed anhydrides and the whole process obtained around 60 grams of crude FTS in 50 working days.

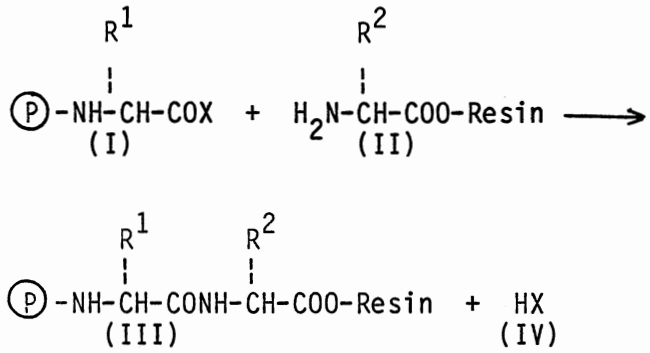
In 1986 a reactor capable of preparing up to 100 grams of purified peptide per synthesis was built in Smith, Kline and French laboratories (13). This facility is designed for a multi-reactor peptide synthesis, capable of synthesizing either four different peptides or larger quantities of one peptide. This is the first systematic study of design considerations for a large scale peptide synthesis reactor, including

agitation of resin slurries, comparison of flow patterns and diffusion between packed and fluidized bed peptide reactor.

Reaction Rate Measurement Techniques

The success of SPPS depends on the completion of deprotection and subsequent addition of each amino acid. This leads to various reaction monitoring techniques. In most cases, the method of detecting reaction completion involves an additional chemical reaction which indicates the presence of the deprotected amine functionality. Those methods include ninhydrin (14), quantitative ninhydrin (15), and the picrylsulfonic acid test (16). A newly developed method, which is different from the above chemistry methods was proposed by Kalbag, et al. (17). The method takes advantage of heat lability of the Boc group which on pyrolysis yields isobutene and carbon dioxide. Pyrolysis of a special resin containing an isopropyl ester moiety with an attached Boc-amino acid residue yields a mixture of propene and isobutene. Quantitative comparison of propene to isobutene by Gas Chromatography indicates the efficiency of both the deprotection and coupling reactions in SPPS. However, these discrete methods have some drawbacks such as a small peptide-resin sample is necessary, a time lag is produced, and the reaction rate information is missed. The most common used method for detecting reaction completeness is the ninhydrin test, but a drastic decrease in sensitivity was observed with increasing chain length (18).

With regard to reaction rate measurement, the coupling reaction in SPPS can be expressed as



(1)

where P represents the protection group, and R^1, R^2 are side chain groups of the amino acid. The reaction can be followed by measuring either the decrease of incoming amino acid derivative, (I), the decrease of free peptide chain (II), the increase of newly incorporated amino acid into (III), or the increase of by product, (IV).

The first attempt at continuous monitoring of the reaction was by Gut, Rudinger and coworkers (19-21). The strategy was measurement and recording of the transmittance of the circulating reagent solution (I) at a suitable wavelength of a spectrophotometer. This permits the changes in concentration of reactant or reaction product to be continuously followed. The coupling reagent was active ester in dimethylformamide (DMF) solution. Five fold excess of active ester was used and pseudo-first order reaction rate kinetics was forced fit to calculate the rate constant, k_1. It was found that k_1 decreases as percent conversion increases.

Another attempt was done by Hodges (22) in 1975. An apparatus was designed to automatically monitor the progress of SPPS by measuring the

total peptide chains after deprotection and the number of unreacted peptide chains (II) after the coupling reaction. The method is based on spectrophotometric picrate method for the determination of resin-bound amino-groups. The picrate monitoring method is accurate and reliable according to the paper, but reaction rate and reaction kinetic parameters were not discussed. In 1984, Merrifield (23) used a simple apparatus for measuring reaction rates in SPPS by spectrophotometrically measuring concentration decreases of the amino acid (I) with usable chromophores such as the Bpoc or Nps protection groups. Coupling rates of Bpoc-amino acid anhydrides with amino acid-resins were listed. Unfortunately, the reaction parameters had not been studied and the limited commercial availability of Bpoc and Nps amino acids made more studies about Boc amino acid reaction rates necessary.

Kinetic Variables

Solid phase peptide synthesis involves a heterogeneous reaction mixture composed of an insoluble resin-bound peptide chain, a soluble activated amino acid derivative and a solvent. It is important to know the role of each component in the process of this reaction.

The discussion of literature can be started from the reactants involved. Both the incoming amino acids and the anchored peptide are important factors. Competition studies between amino acids (24-25) have shown that isoleucine, valine and O-benzyl-threonine have substantially lower reactivities than other amino acids. This competition reaction was done on 1% divinylbenzene cross-linked polystyrene resin and the lower reactivity of the above amino acids contributed to their steric

hindrance. On the other hand, amino acids protected by groups containing one or two urethane structures all appeared to have similar reactivities. More should be done on the effect of steric hindrance on reactivity. One might study the coupling reaction rate of amino acids polymer resin with different degrees of cross-linking in order to see more physical limitation of the reaction environment. The size of the incoming amino acid or peptide fragment in the condensation reaction could also affect the kinetics of SPPS.

The role of the anchored peptide in reactivity and kinetics has also received increasing attention. Its influence is interrelated with the solvation properties of the solvent and resin. Several difficult synthesis problems, originally thought to be resin induced, have been shown to be due to solvation problems (26). Kent gives an interesting example of this in the synthesis of two tetrapeptides, Boc-Tyr(clBzl)-Ile-Asn-Gly and Boc-Tyr(clBzl)-Ile-Ala-Glu(OBzl), in which all variables were held constant except for the two interior residues. The resulting rates were quite different. About 99% of the Ile-Ala-Glu(OBzl) resin had reacted within 30 minutes while about 33% of the other resin remained unreacted. NMR spectra revealed intermolecular aggregation and decreased mobility, similar to precipitation, in the slower reacting peptide-resin. While using DMF, a better solvating solvent which can accept hydrogen bonds and displace the peptide-peptide interaction, could completely eliminate these effects. The resin employed can also influence solvation. It has been shown that the peptide and resin exhibit a mutual solvating effect. Therefore, a better solvating resin, such as Pam resin and a suitable solvent as discussed previously in SPP chemistry, could improve reaction rates. In this aspect, Merrifield has

comprehensive data of solvation properties of different resin in solvents (27).

Several other aspects of the importance of the anchored peptide on SPPS-kinetics should also be considered. The aggregates described above occur most often between 4-18 residues (28), suggesting chain length involvement. The secondary structure of peptide chain α-helix or β-sheet, may also influence kinetics and the impact of secondary structure on kinetics has not received attention, although peptide mobility has been shown to be similar inside the resin and in solution (29-30). The reactivity of the terminal amino group on the peptide must also have a significant effect on reaction rates. The peptide bond is formed by a nucleophilic reaction of the terminal amine and pKa values are directly representative of reactivity. A study done on one sample peptide showed an increase of pKa over the first four residues which is equivalent to acylation rates increasing by a factor of 30 (31).

A study on general kinetic characteristics of reaction involving a cross-linked polymer reagent was done by Pan and Morawetz (32). They proposed that the deviation from first order reaction rate in polymer support reflects the variation of local polarity and reactivity of reactive groups attached to cross-linked polymer. This explanation was concluded from the studies of acylation of aromatic amine residues in cross-linked polymer by acetic and butyric anhydrides, and the authors still consider the explanation was not proven yet. However, the technique that converts a fluorescing reagent to a nonfluorescing reaction product is worthy of further development of kinetic analysis on solid/solvent reaction.

In heterogeneous reaction, mass transfer of reagent sometimes is the rate limiting step. This is not the case in SPPS. Mass transfer into the (1% to 2% DVB) resin beads has generally been accepted as not influencing rates (23). High peptide and resin mobilities in most cases indicate that internal diffusion is not rate limiting (29-30). A more specific example given by Merrifield, in the reaction of $(Bpoc\text{-}Phe)_2O$ with val-resin, the rate of transfer of Boc-Phe into the beads appeared to be in excess of 6×10^{-5} mmol/cm^2 sec, and the new $(Bpoc\text{-}Phe)_2O$ inside the beads must be replenished at a rate of 0.4×10^{-5} mmol/cm^2 sec according to the reaction rate. Therefore, mass transfer does not seem to limit the reaction rate. Besides, internal volume and reactive space inside the resin actually increases with peptide chain (23). This fact also helped reduce concerns about both a maximum chain length and dramatic increase in steric hindrance. All of these results are based on the use of a solvent which provides adequate swelling of the resin network. On the other hand, the solvent's properties, polarity, dielectric constant, etc, might make hydrogen bonding between reagents and peptide-resin. This could cause the reaction rate measurement to fail.

Previous Experimental Work

Actual kinetic data on classical SPPS is limited, and industry is bringing the technology into line without full knowledge of reaction kinetics. Data is often available for different reaction schemes. No kinetic data, rate constants or reaction half-times, were located for the classical SPPS method. For this reason, only relative and qualitative comparisons will be possible in this study.

Several different types of data on SPPS kinetics are available. First, rate constants and half-times for other schemes are available. These include work on: linear polystyrene resins with active esters and DCC coupling (33), Nps protected active esters and Boc protected thiolesters (20), and Bpoc anhydrides (23). Another source of qualitative information is the use of competitive reactions (24-25). In these experiments an equimolar mixture of several different amino acid anhydrides is added to peptide-resin. Analysis of the resulting peptides reveals relative reactivity between different amino acids. A final source of general kinetic information is the overall reaction time used, combined with yield (34).

However, most of the data presented on the attachment of one amino acid derivative to resin has only one or two amino acid residues. The reaction rates on a series of amino acids attachment to a peptide with increasing chain length have not been studied. Although the high reaction temperature has been used to elevate the reaction rate (35), the effect of temperature on reaction rate is still missed. Furthermore, the comparison between different percent cross-linked polystyrene in coupling rate has not been done. Thus, the above mentioned variables as well as different excess mole ratio will be addressed in this study.

CHAPTER III

EXPERIMENTAL APPARATUS

Several important considerations were involved in the development of the experimental apparatus. The major concern, and the biggest difference between this design and most other schemes, was the desire to have a continuous stream for nondestructive monitoring of the reaction dynamics, and an alternative to the standard mixing, rocking, procedure is also needed. Other factors which influenced the final design were the chemicals involved, the small volumes to be used and the general ease of operation. Figure 2 illustrates the final scheme used. A description of the experimental apparatus is given below.

Reactor

A closed water-jacketed reactor with a coarse glass frit bottom is the major feature of the experimental set-up. The capacity of the reactor is 100 ml. Temperature was controlled by a constant temperature bath and read by a thermocouple inserted into the reactor glass wall. Mixing was accomplished by a speed controlled stirrer suspended from the top to avoid bead damage. A closed reactor is necessary for maintaining the constant volume of reaction mixture during measurement. The volume under the frit bottom is reduced to a minimum in order to cut down the reagent volume outside of the reactor. Silanization of glassware before synthesis is needed to prevent basic or hydrophobic peptide-resin from

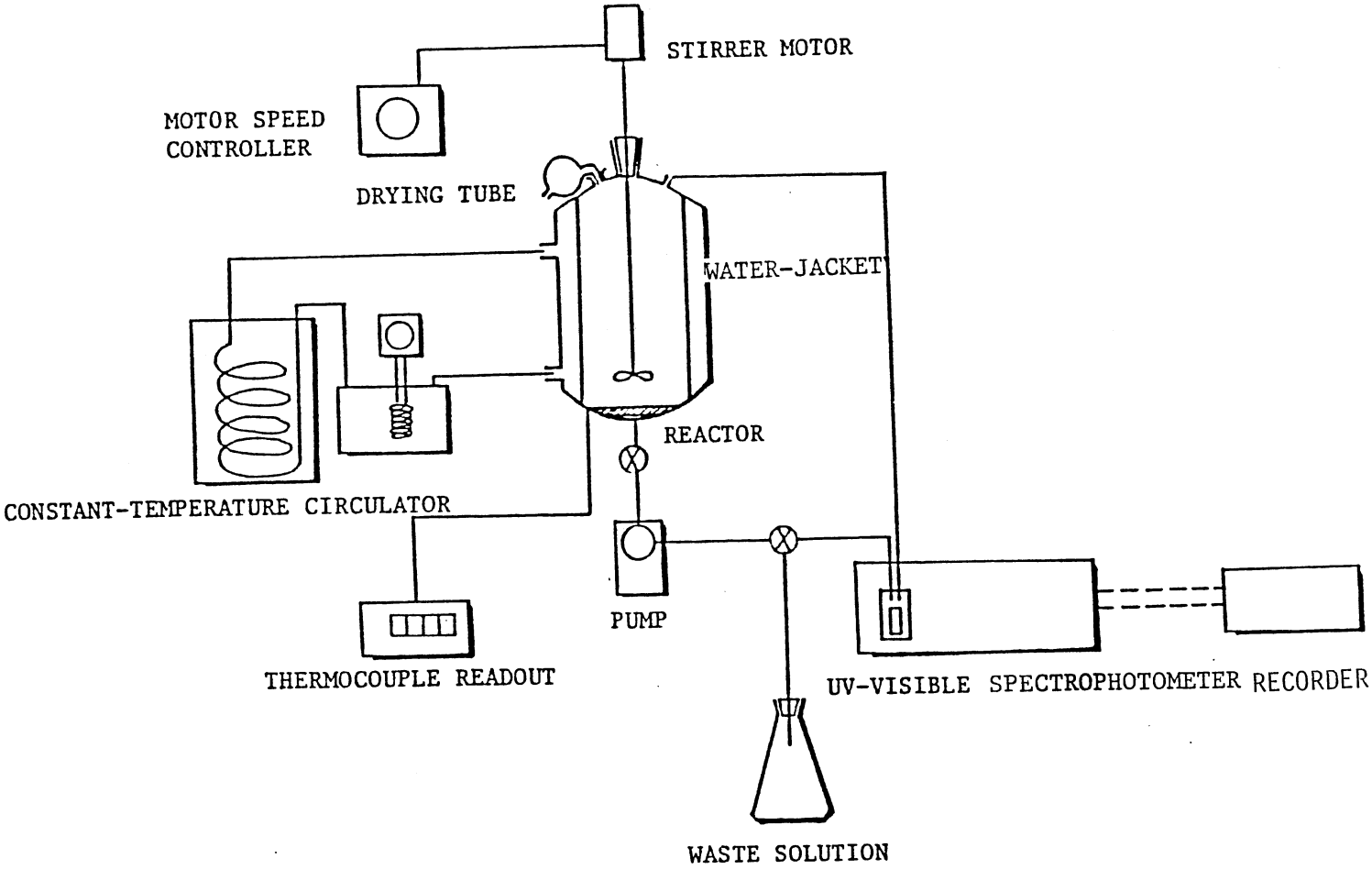


Figure 2. The Experimental Set-up for the Kinetic Study of Solid-Phase Peptide Synthesis

adhering to the reactor wall. However, polymer beads attached to the reactor wall can be washed into the bulk solution by manually introducing the appropriate solvent through the reactor input. Since the symmetrical anhydride coupling was the major reaction scheme, a drying tube with $CaCl_2$ is used to minimize anhydride hydrolysis during the synthesis.

Monitoring Loop

The monitoring loop consisted of a three-way stopcock, a piston pump, a flow through cell and an Ultraviolet-Visible spectrophotometer, all connected by about 2 ft of 1/16 in FEP-teflon tubing. The total volume outside of the reactor is about 3 ml. The stopcock directs flow either to waste or to the circulating loop. A piston pump, model RHSYICKC, from Fluids Metering Inc., provides circulation. On the settings used, this pump gives flow rates between 10 and 25 ml/min and results in a lag time between reactor and detector of approximately 5-10 seconds. To achieve continuous ultraviolet monitoring, the solution is pumped through a quartz flow cell. The cell has a 10 mm light path and a capacity of 0.6 ml. An available Perkin-Elmer Lambda 3 UV/VIS spectrophotometer measures absorbance. This data was recorded with a Perkin-Elmer 561 recorder. After leaving the flow cell this reactant solution returns to the reactor.

Mixing Device

Several design features are also involved in the resin mixing procedures. At the start of this study, bubbling nitrogen through the reactor was used to achieve mixing. Due to the high vapor pressure of

reaction solvent, DCM, the nitrogen was bubbled through a separate column of solvent to provide presaturation. The saturated nitrogen then passed to the reactor. A cold-trap condenser removes solvent from the leaving nitrogen stream. This design caused the reaction mixture volume to change which gave UV-absorbance deviations making the spectrum analysis impossible. Currently, a Cole-Parmer speed-controlled mixer is used, the closed system eliminates the solvent change problem and provides different speeds of mixing which is an additional reaction parameter.

A few additional components are included in the complete design. First, the temperature control was done by Haake which can control temperature to $\pm$ $1^{o}C$. A solid state motor speed controller provides a constant speed of mixing rate. Finally, all of the equipment was placed in a walk-in enclosed hood, vented to outside the building.

CHAPTER IV

EXPERIMENTAL PROCEDURE

Some preliminary work is needed before synthesis of peptides can begin. The preliminary experimentation mainly consists of preparing calibration curves for both the amino acids and their symmetrical anhydrides. A series of samples are made by diluting a solution of known amino acid or anhydride concentration. Ultraviolet absorbance of each sample is then taken at numerous wavelengths. Note here that the maximum UV absorbance wavelength is not always the wavelength used at the measurement during the synthesis. Sometimes the concentration of symmetrical anhydride and amino acid under investigation makes the UV-absorbance out of range. Although the maximum absorbance can be used by arranging the light path length of the flow cell, this was not done in this study. By plotting absorbance vs concentration at several wavelengths, calibration curves with the proper absorbance scale over a range of concentrations are obtained. A detailed description of anhydride preparation and spectrophotometer use are given in steps 11-15 of the following peptide synthesis procedures.

Peptide Synthesis Procedure

One to two percent divinylbenzene cross-linked polystyrene resin with the first amino acid residue already attached and analyzed is used exclusively in this study. Due to this fact, many common resin

preparation steps are omitted. Stewart and Young provide detailed experimental procedures for these reactions and tests (11). After a weighed sample of resin is placed in the reactor, the repetitive synthesis steps outlined by Stewart and Young are illustrated in Table I.

TABLE I

SOLID PHASE PEPTIDE SYNTHESIS STEPS WITH SYMMETRICAL ANHYDRIDE COUPLING

Step	Reagent	Volume[a](ML)	Time[b](min)
1	DCM	15	5
2	DCM wash (3 times)	15	1.5
3a	TFA/DCM	15	1.5
3b	TFA/DCM	15	30
5	DCM wash (6 times)	15	1.5
6	TEA/DCM wash (2 times)	15	1.5
7	DCM wash (6 times)	15	1.5
11-13	Symmetrical Anhydride in DCM	15	-[c]
15	DCM wash (3 times)	15	1.5

a. The volume of solvent depends on the vessel used and the mass of resin. The volume here is based on 1 grams of resin used.
b. Time of each wash and it is approximate.
c. Time needed-until U.V. absorbance is constant.

A detailed description of these reactions, based on a synthesis with one gram of resin, is given below.

1. The Boc-amyl-resin is allowed to swell in 15 mls DCM for 5 minutes before the stepwise synthesis. After the synthesis the peptide resin may be left overnight at this stage suspended in DCM.

2. Wash the resin with 15 mls DCM 3 times. Each wash is approximately 1.5 minutes in length. During these wash and all following washes, the resin is mixed by the stirrer and after the resin is all dispersed and suspended, the wash solvent is pumped out.

3. Deprotection is accomplished using 30 mls of a 1:3 solution of trifluoroacetic acid: DCM which also contains a small amount of Indole (less than 1 mg/ml). Indole is essential if tryptophan is present in the peptide. But, indole is always included to prevent any oxidative effect of the TFA on the peptide and to scavenge harmful contaminants in the TFA. The reagent is allowed to stand overnight before use. Half of the reagent (15 mls) is added to the resin for a 1.5 minute pretreatment, drain and the remaining solution add to the resin for 30 minutes. Step 4 can be started during this half hour.

4. The Boc-amino acid anhydride is prepared outside of the reactor in a small vial. Weigh out an amount of Boc-amino acid which give a desired anhydride to resin sites mole ratio. Dissolve the amino acid in minimum amounts of DCM. For less soluble amino acids, such as tryptophan, dissolve in the minimum volume of DMF and then add DCM to desired volume. The amino acid solution and a 1 M DCC (0.206 g DCC/ml DCM) are then cooled to $0^{o}C$. After being cooled, DCC solution is added to the amino acid solution such that the number of moles of DCC is half the moles of amino acid, i.e. 1:2 mole ratio DCC: amino acid. The

solution is then kept at 0°C for at least one hour with occasional shaking.

5. Deprotection is followed by 6 DCM washes each of 15 mls. Additional washes should be added if the resin still retains purple coloring from the indole. Usually the longer the TFA/DCM with indole solution prepared, the deeper the purple color of the resin. The resin can be left overnight after this step. The final peptide-resin should be left deprotected.

6. The peptide is next neutralized by two treatments with a 1:9 solution of triethylamine (TEA):DCM. Each rinse is 15 mls and should be about 1.5 minutes in duration. The remaining steps should be carried out as quickly as possible.

7. Neutralization is followed by 6 DCM washes, 15 ml each. During these washes, preparations for the following steps should be made. This includes getting the spectrophotometer ready (Step 8) and having prepared the known concentration of the symmetrical anhydride solution.

8. With both channels completely empty, the ultraviolet spectrophotometer is turned on as instructed in the spectrophotometer users' manual. This should be done 1 hr before measuring because of time needed for warming-up the machine and background light correction. Set the UV wavelength to the appropriate value from the calibration curve. Clean both cells and fill with DCM and insert into the U.V. compartment, zero the absorbance. At this point, the front cell is ready for measuring the initial absorbance reading of symmetrical anhydride solution. This is done in order to check with the calibration data and give the initial point of UV-absorbance curve, since from experience, the initial point usually doesn't show because the lag phase caused by

the pump, initial mixing of anhydride with DCM used to preswell resin, and the jump of UV absorbance caused by air bubble in the TFL-teflon tubing.

9. Turn on the chart recorder. Adjust the zero setting, chart paper speed, recording scale and set remote control on.

10. After the six washes, dry the resin by draining as much as possible. Use the pump to remove solvent from tubing and stopcock. To have resin in swelled state and to avoid preferential solvent absorption, accurately measure amount of DCM (about half of reaction volume) was added to resin.

11. To prepare the anhydride for coupling, the dicyclohexylurea (DCU) precipitate must be removed. First, the anhydride is allowed to warm to room temperature, this prevents moisture from condensing into the anhydride solution. Anhydides are very water sensitive and should be exposed to air as little as possible. Therefore, the following should be done only after all other preparations have been made. Quickly filter the anhydride solution through filter paper with fast filter speed (Whatman #4). Rinse the vial and DCU with DCM and bring the concentration of anhydride to the desired value. Empty the front standard cell and fill with the initial concentration of symmetrical anhydride solution, record the initial absorbance reading.

12. Insert the clean flow cell which has already been connected to the reactor as shown in Figure 2. Pump the DCM in the reactor through the whole monitoring loop. Zero the absorbance reading. At this point, the environment for measuring the absorbance is the same as step 8 and the absorbance caused by DCM is zeroed. When this is all set, let the recorder paper start running. Although the UV cutoff of DCM is 233 nm,

which is much lower than the working wavelength, this act can take the noise of absorbance caused by cell or DCM out of consideration.

13. As simultaneously as possible, dump anhydride into the reactor. At this time, the stirrer speed and the reaction temperature are all at desired value. Press on the UV run key which starts the measuring of absorbance.

14. No change in U.V. absorbance for about 5 minutes is assumed to represent completion of reaction. Drain the reaction solution, clear the monitoring loop by pumping through DCM. Shut everything off. Rinse pump with ethanol.

15. Wash resin with 15 mls of DCM 3 times. If DMF was used for solubility reasons, insert a wash with 15 mls DMF before the DCM washes. Finally, the resin can be left suspended in DCM before the next synthesis step.

16. Test for completeness of reaction. A modified version of Stahl, Walter and Smith's test (36) gives good qualitative results. A 1% solution of picrylsulfonic acid is prepared in fresh DMF. Approximately a 2 mg sample is placed in a very small test tube and two drops of the reagent and two drops of 10% diisopropylethylamine in chloroform solution is added. After 10 minutes at room temperature, 1 ml of ethanol is added and the resin beads are viewed through a magnifier. All colors should be associated with the beads. A positive test (little coupling) is indicated by a bright red color (approximately 0.5 mmol/g) to a faint yellow for almost complete coupling (0.001 mmol/g).

17. The reading in step 11 is the initial absorbance reading, and the final reading in step 14 are supplied as the bases for 100% reaction as if the step 16 had a negative, complete coupling result.

CHAPTER V

RESULTS

In this section, the results of the synthesis of homopolyserine and homopolyphenylalanine in various reaction conditions and the synthesis of Bradykinin will be presented. Emphases will be put on qualitative analysis of the effect of reaction parameters on the reaction rates. In addition, various ways of presenting data will be tried.

The first experimental results needed are the calibration curves for both the free amino acids and their anhydrides. The details of obtaining this information are discussed in Dietrich's thesis (37), and the calibration curve equations are listed in Appendix A.

These calibration curves were prepared at a variety of wavelengths to determine the best operating conditions for the analysis of amino acids in the presence of chlorinated hydrocarbon solvent. Due to the concentrations of symmetrical anhydrides used in the reactor the UV wavelength could not be set at the optimum detection point. Furthermore, it should be noted that the absorptivities of amino acids and their anhydrides are not the same or in the relationship of 2:1 because of the number of UV-absorbing functional groups. As a result, the analysis of UV-absorbance should take both symmetrical anhydride and free acid (the coupling byproduct) into account.

Table II lists the peptides synthesized and the specifications of synthesis and reaction materials.

TABLE II

LIST OF PEPTIDES SYNTHESIZED

EXPT.	Peptide	Reaction Temp.^{o}C	Mixing Rate(RPM)	M.R.[a]	%CL[b]	Loading[c] (mmol/g)
Peptide I	Poly(ser)$_{10}$	26	200	1.5	2	0.2
Peptide II	Poly(Phe)$_{10}$	26	200	1.5	2	0.7
Peptide III	Poly(ser)$_{10}$	14	200	1.5	2	0.2
Peptide IV	Poly(Phe)$_{10}$	14	200	1.5	2	0.7
Peptide V	Poly(Phe)$_{10}$	26	200	1.2	2	0.7
Peptide VI	Poly(Phe)$_{10}$	26	100	1.5	2	0.7
Peptide VII	Poly(Phe)$_{10}$	26	200	1.5	1	0.43
Peptide VIII	BradyKinin	26	200	1.5	1	0.45

a. Mole Ratio, Amino Acid Symmetrical Anhydride (C_X) to amino terminus of peptide-resin (C_A).
b. Percent divinylbenzene crosslinking of polystyrene resin.
c. ± 2% of loading was reported by supplier, SIGMA Chemical Company.

Commerically available divinylbenzene (DVB) polystyrene copolymer resins with the first amino acid attached were used as starting materials for synthesis. The dry resin size was 200-400 mesh, and the loading of the first amino acid on the resin varies, which is specified by the supplier, SIGMA Chemical Company. Also, N-α-t-Boc-L-phenylalanine and N-α-t-Boc-O-benzly-L-serine were supplied by Chemical Dynamics Corporation. The reagents used in the deprotection step was 25 volume % trifluoroacetic acid (TFA) in DCM, and the reaction medium was DCM. The relative volume of resin and external solution is about 1:6

for 1.3 g resin in 30 ml reaction volume, depending on the peptide content. For a detailed description of experiment procedures and reagent volume refer to the EXPERIMENTAL PROCEDURE. A blank run, with the terminal residue still blocked, was performed to understand the mass transfer of anhydride and the mixing effect of volume change at the time of symmetrical anhydride addition. The UV-absorbance curve was traced directly from recorder paper, Figure 3. The bump represents a higher concentration of anhydride at the beginning, which might be caused by poor mixing between the anhydride solution and the solvent used to preswell the resin, or partially because of the delay in anhydride distribution between the bulk and reaction phases. The contribution from the partition of symmetrical anhydride between bulk phase and resin is negligible since the equilibrium of anhydride between phases is reached within 2 seconds as determined by NMR (38). However, for data analysis simultaneous mass transfer and reaction was assumed during the initial period in which anhydride penetrates the resin. This assumption will be discussed in detail in Chapter VI.

Kinetic Data

Peptide Chain Length

Syntheses of homopolyserine and homopolyphenylalanine to residue number 10 were conducted to determine the influence of peptide chain length on the coupling rate (Peptide I, II and VII). Figures 4 and 5 illustrated that the reaction rate decreases as the chain length increases. (Data were collected for every attachment, but only every other set is plotted to avoid clutter). This phenomenom is true for both serine and phenylalanine in various reaction parameters, like

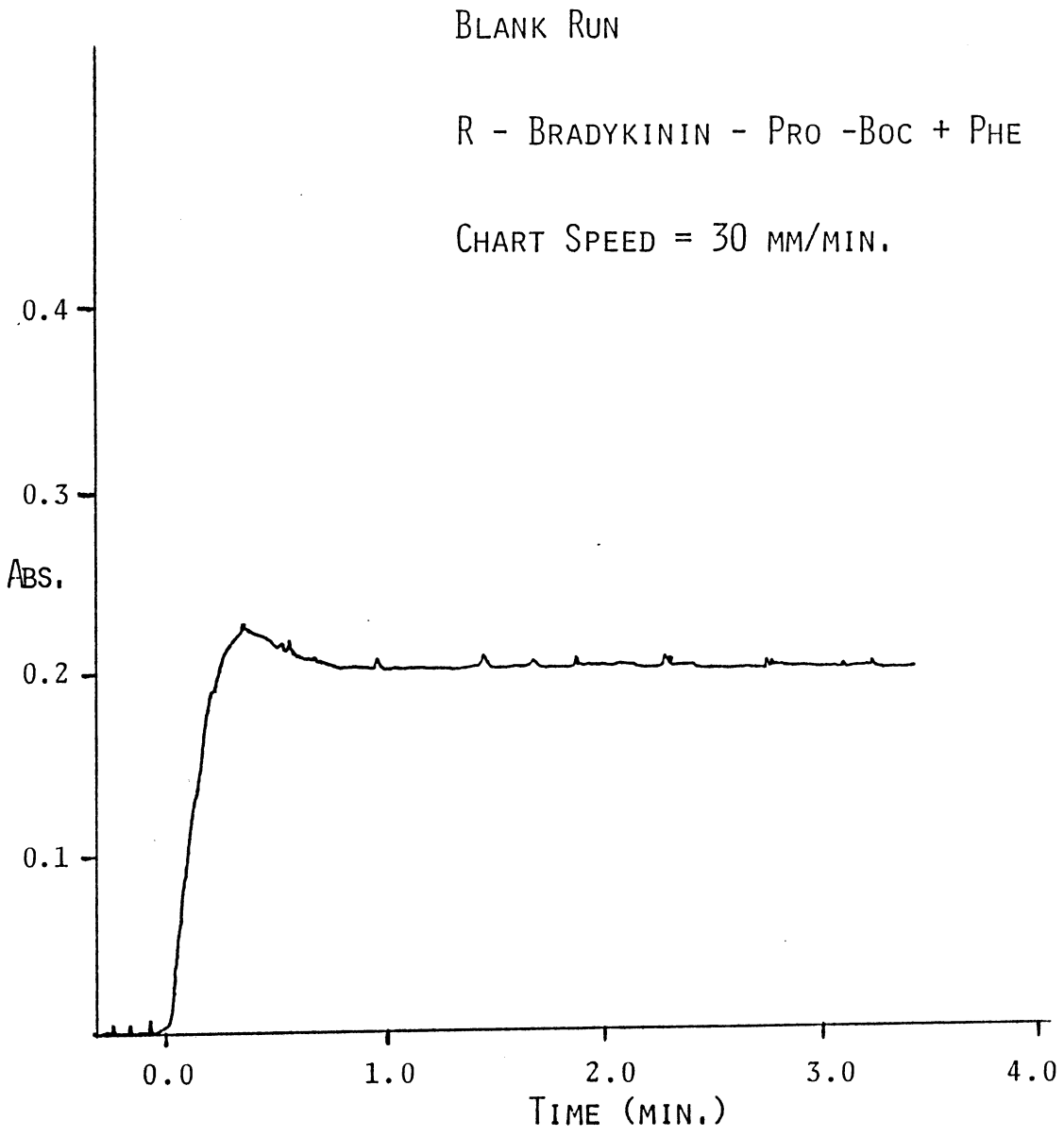


Figure 3. Absorbance Curve of Blank Run

temperature and initial resin loading, with the reaction rate of serine being faster. This different reactivity agrees with the competitive experiments performed by Ragnarsson, et al. (25). Note that the loading for Boc amino acid on chloromethylated polystyrene (2% divinylbenzene cross-linked) was 0.7 mmol/g resin for Phe and 0.2 mmol/g for Ser. Also, 99.5% plus acylation of the amino terminal was indicated by the picrylsulfonic acid method for each coupling reaction. The ester linking the peptide to the resin in the classical SPPS system is slightly labile to the reagents normally used for removal of Boc groups at each step of synthesis, when 25% TFA in DCM is used for deprotection, this loss may be as high as 1% per deprotection step (11). While this loss may not be of serious consequence in the synthesis of short peptides, it is not acceptable for synthesis of long peptides. This kinetic study is not capable of quantitatively detecting this loss without supporting information by, for example, the picrysulfonic acid method.

The initial concentration of symmetrical anhydride was 1.5×10^{-2} mol/l for Ser and 4.602×10^{-2} mol/l for Phe at a reaction volume of 30 ml. These are in the concentration range where Merrifield (23) indicates that mass transfer is not a limiting factor in coupling reactions between amino acyl-resin with anhydride. Table III presents the reaction time versus per cent conversion of active sites of peptidyl-resin.

TABLE III

REACTION TIME VS CONVERSION OF AMINO TERMINUS
(PEPTIDE I (SER))

Time(sec.)	n[a]	1	3	5	7	9
$t_{0.5}$		[b]10	[b]12	[b]20	30	40
$t_{0.75}$		35	60	75	123	171
$t_{0.9}$		92	123	180	321	480

TABLE IV

REACTION TIME VS. CONVERSION OF AMINO TERMINUS
(PEPTIDE II (PHE))

Time(sec.)	n[a]	1	3	5	7	9
$t_{0.5}$		[b]20	[b]26	51	71	92
$t_{0.75}$		76	102	219	250	424
$t_{0.9}$		235	260	510	610	1060

a. number of residues on peptide
b. data was obtained through interpolation

Based on the ideal second order rate constant, proposed by Merrifield (23), for the coupling of Bpoc-Leu symmetrical anhydride with Ala resin the time for 99% reaction completion was estimated to be 14 seconds. Although a different protection group of amino acid was discussed, the order of magnitude of reaction times are compatible.

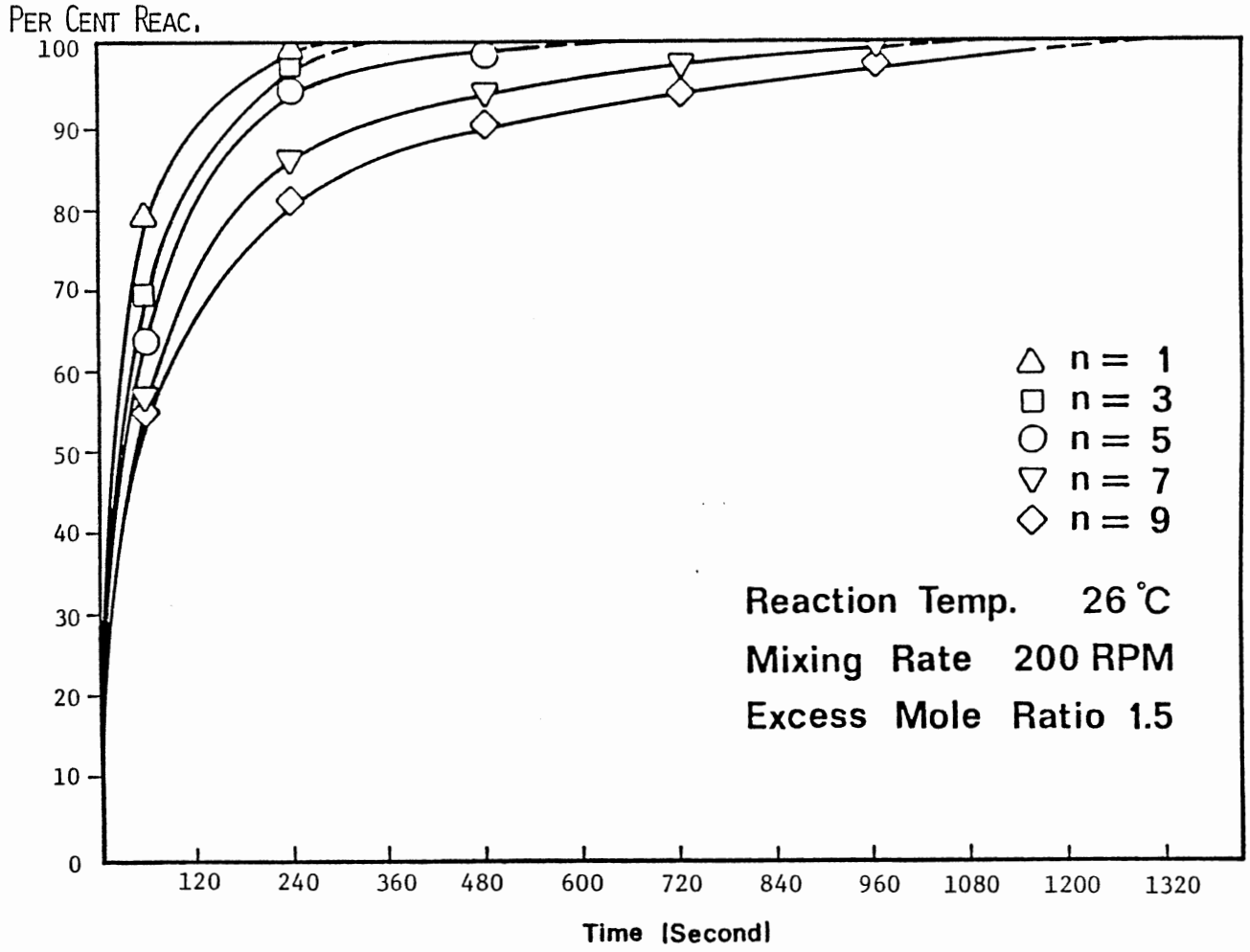


Figure 4. Percent Reaction of Amino Terminus Versus Time
(Polyserine, Peptide I)

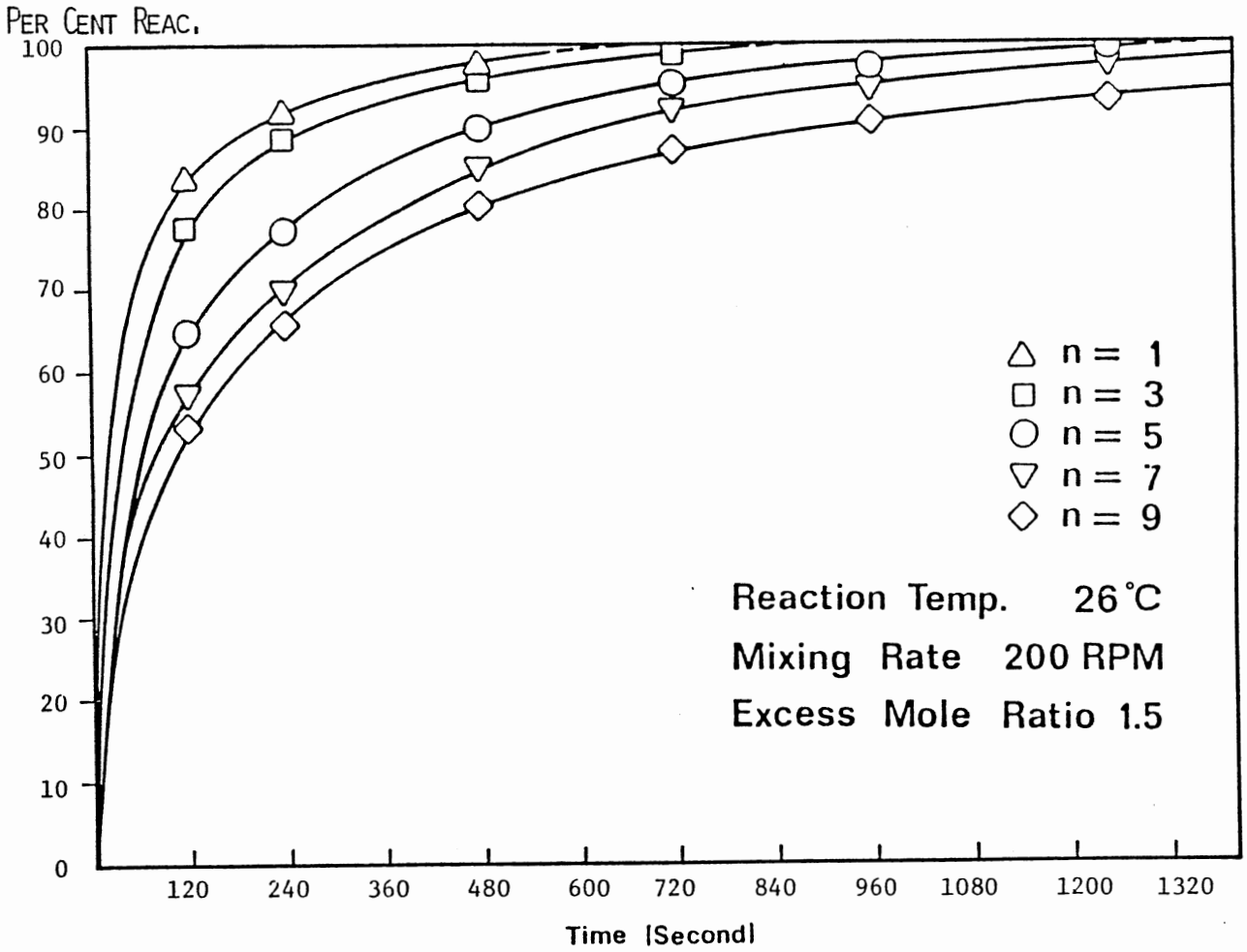


Figure 5. Percent Reaction of Amino Terminus Versus Time
(Polyphenylalanine, Peptide II)

Merrifield did not verify the experimental results with his estimation. From the general observations of this study, the kinetics tend to deviate from ideal second order reaction at the latter stage of synthesis. Thus, the estimated conversion time by Merrifield may be shorter than the actual time required.

Reaction Temperature

Experiments of peptides I and II were repeated at a lower reaction temperature, 14°C (Peptide III and IV). The results are shown in Figures 6 and 7. Similar chain length effects were observed and, by comparing with Figures 4 and 6, the lower temperature lowered the reaction rate. This effect was enhanced as the peptide chain length increases. Tables V and VI list the times required for different active site conversions.

TABLE V

REACTION TIME VS. CONVERSION OF AMINO TERMINUS
(PEPTIDE III (SER))

Time(sec.)	n[a]	1	3	5	7	9
$t_{0.5}$		[b]14	[b]18	35	51	69
$t_{0.75}$		40	50	107	185	328
$t_{0.9}$		127	178	249	460	833

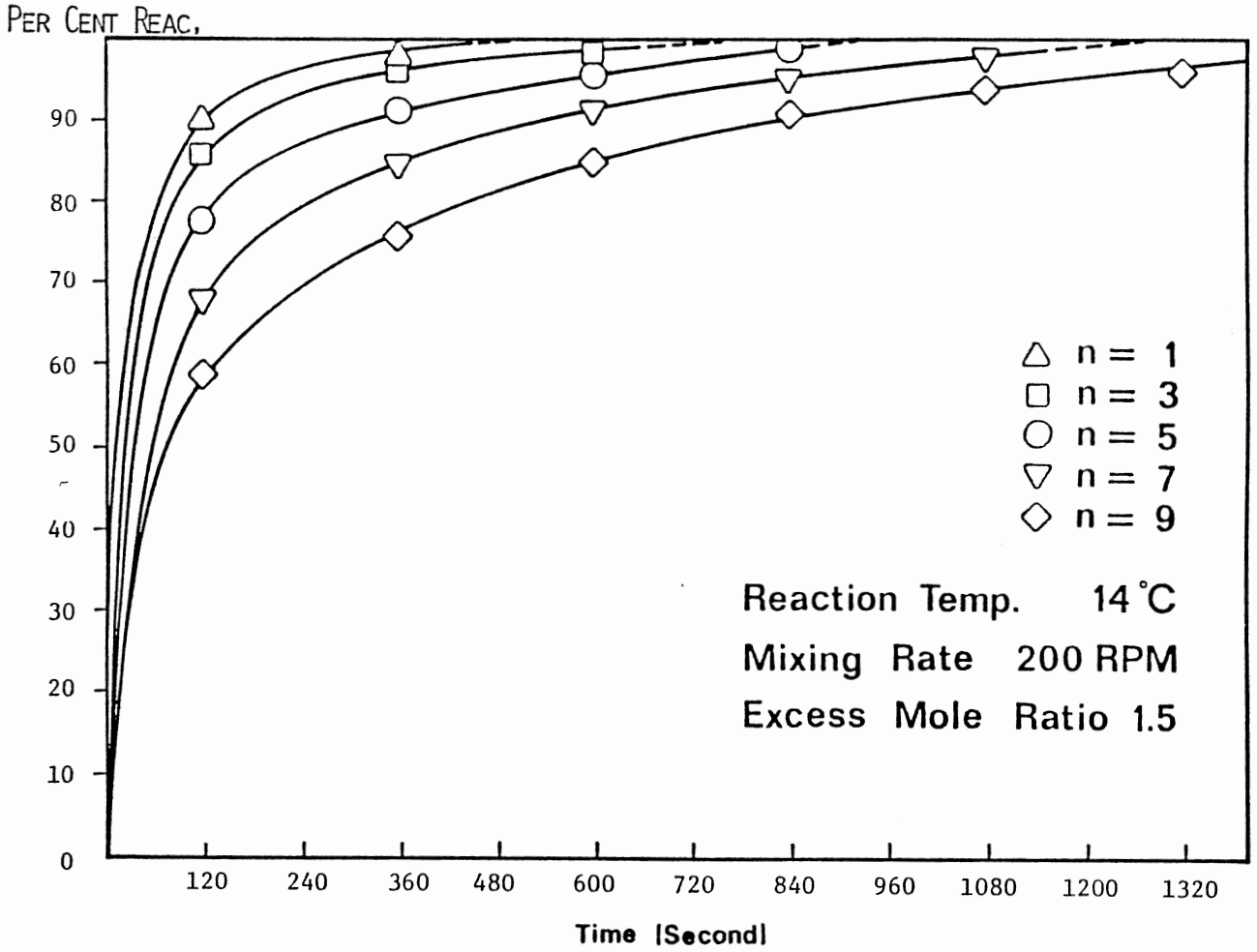


Figure 6. Percent Reaction of Amino Terminus Versus Time
(Polysersine, Peptide III)

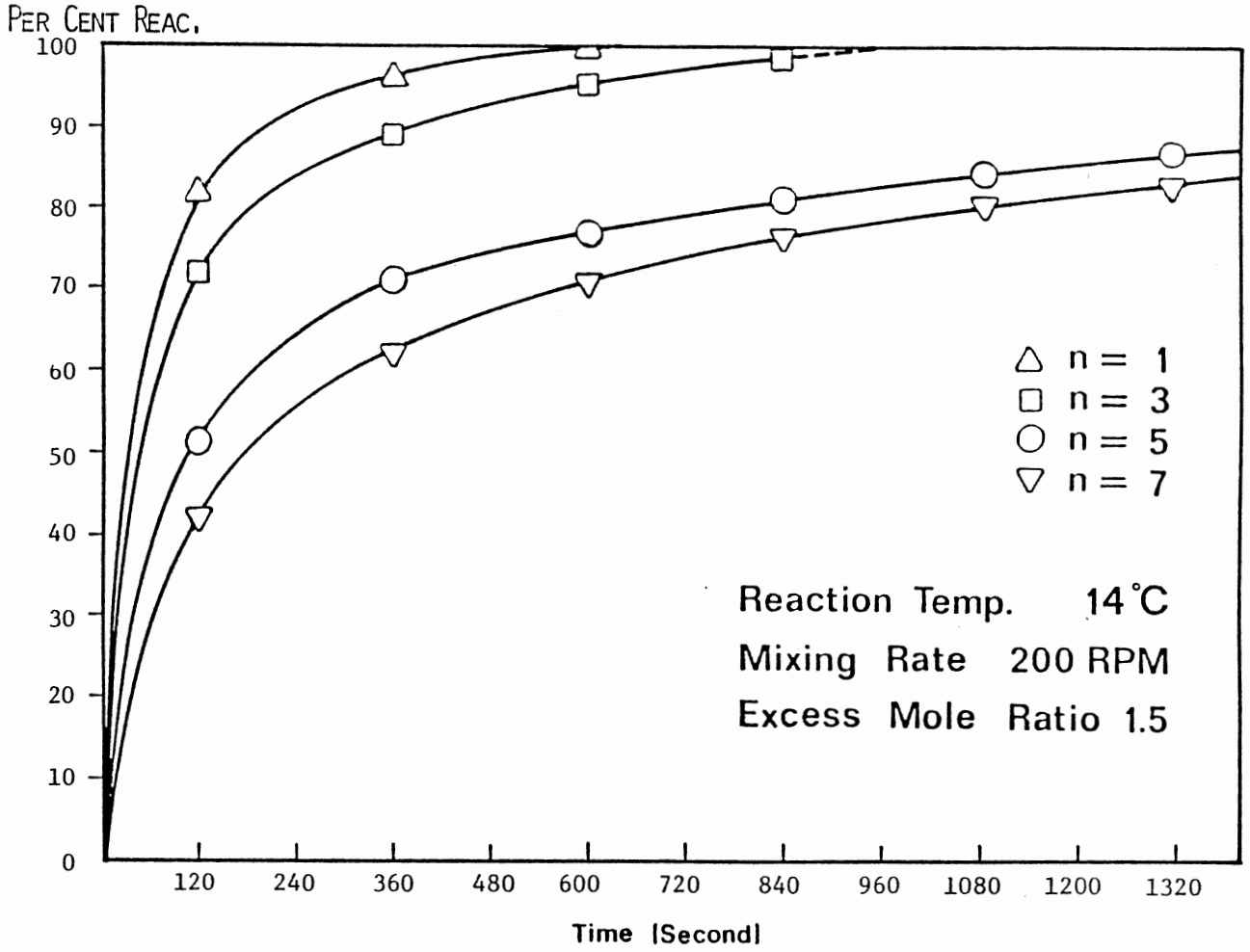


Figure 7. Percent Reaction of Amino Terminus Versus Time
(Polyphenylalanine, Peptide IV)

TABLE VI

REACTION TIME VS. CONVERSION OF AMINO TERMINUS (PEPTIDE IV (PHE))

Time(sec.)	n[a]	1	3	5	7	9
$t_{0.5}$		28	45	122	178	230
$t_{0.75}$		93	142	490	665	935
$t_{0.90}$		218	360	1305	1550	2170

a. number of residue on peptide
b. data was obtained through interpolation

Mixing Rate

Mixing is required to have a uniform distribution of reagents in the reaction mixture. For heterogeneous reactions mixing reduces film mass transfer resistance. Two mixing rates (100 and 200 RPM) were used for the formation of polyphenylalanine (Peptide II and VI) (see Figures 5 and 8). These mixing rates did not affect the coupling rate indicating that film transfer resistance is not a rate limiting step in these experiments. These two mixing rates provide enough shear around the resin particles to limit diffusion resistances to the resin reaction sites. Film diffusion resistance may be minimal on resin sizes of 35-100 microns (23), this introduces a curiosity about the relationship among time, radius and conversion. This relation can give insight of reaction control scheme in heterogeneous reaction. However, a wider range of mixing rates is required to confirm the effect of film transfer resistance. Furthermore, the mixing must produce little or no vortexing

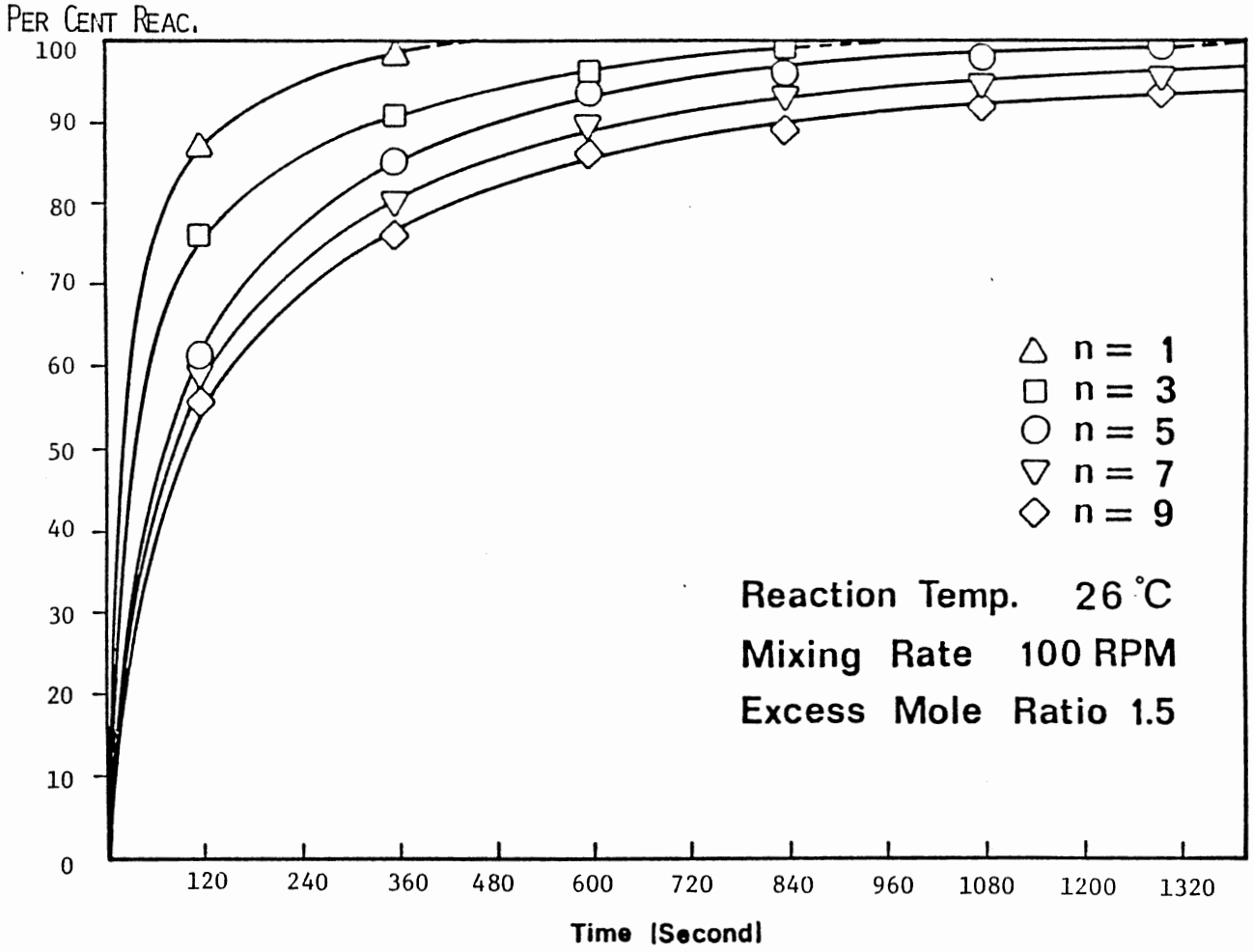


Figure 8. Percent Reaction of Amino Terminus Versus Time
(Polyphenylalanine, Peptide VI)

to prevent resin from adhering to the reactor wall not contacted by the bulk phase of the reaction medium. Also, the fragility of swollen polystyrene in DCM also limits the range of mixing rates.

Mole Ratio

The mole ratios of 1.5 and 1.2, symmetrical anhydride to reactive site or amino terminus, were used for polyphenylalanine synthesis (Peptide II and V) (see Figures 5 and 9). Figure 9 shows a minor decrease in reaction rate for the 1.2 mole ratio at the early stage of peptide synthesis ($n \leq 4$), but a significant reduction is observed with growing peptide chain. This shows the reaction control mechanism may be interrelated with chain length. More discussion is in Chapter VI.

TABLE VII

COMPARISONS OF REACTION TIME WITH DIFFERENT MOLE RATIOS OF SYMMETRICAL ANHYDRIDE TO REACTION SITES IN THE SYNTHESIS OF POLYPHENYLALANINE

Peptide	Mole Ratio	n	1	3	5	7	9
Peptide II(Phe)	1.5	$t_{0.5}$	20	26	51	71	92
Peptide V(Phe)	1.2	$t_{0.5}$	23	40	87	215	290
Peptide II(Phe)	1.5	$t_{0.9}$	235	260	510	610	1211
Peptide V(Phe)	1.2	$t_{0.9}$	185	242	610	2880	4020

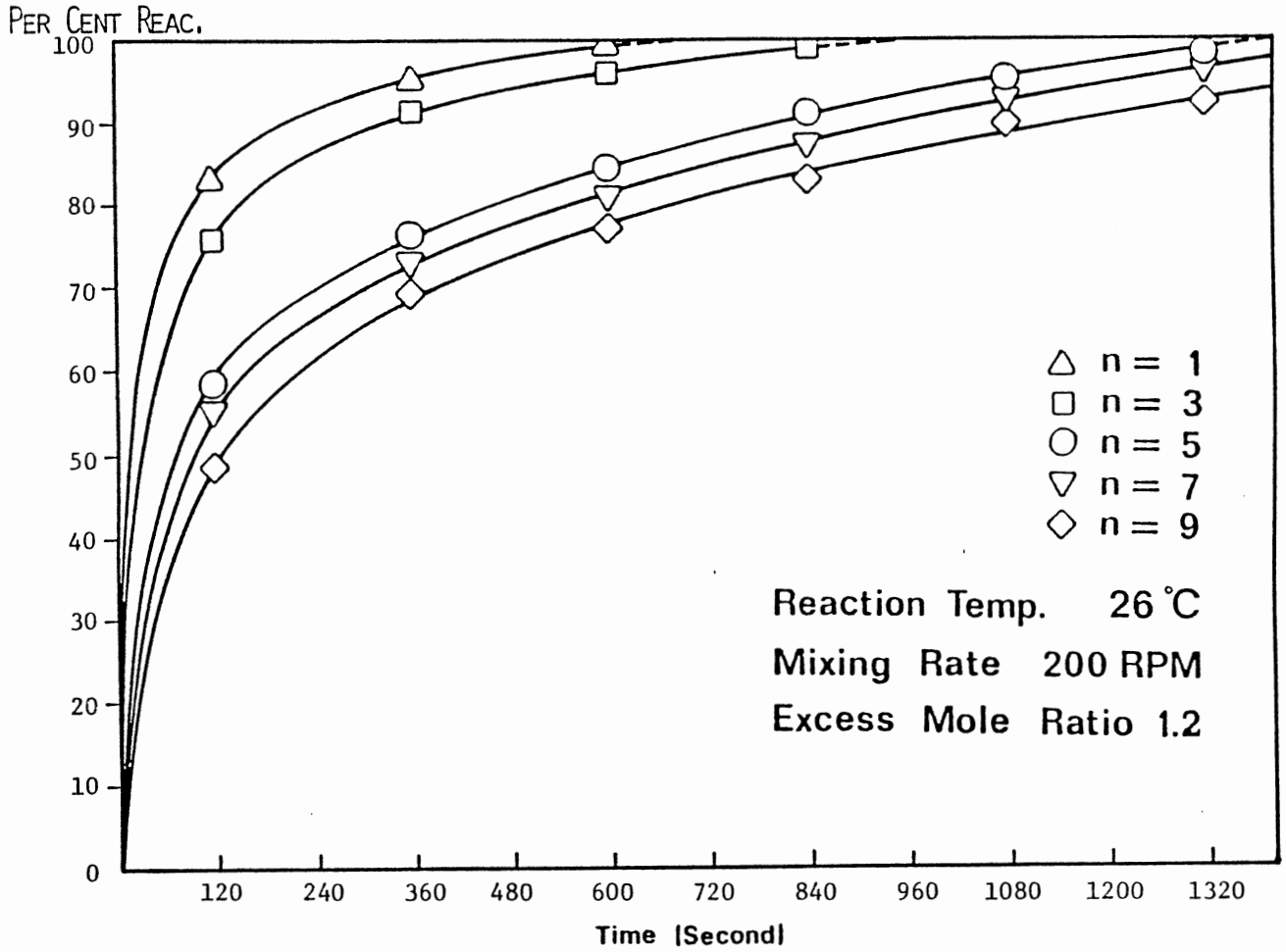


Figure 9. Percent Reaction of Amino Terminus Versus Time
(Polyphenylalanine, Peptide V)

Polymer Support

Polystyrene with 1% divinylbenzene cross-linking with the first phenylalanine attached was used as starting material (0.43 mmol/g resin) in the synthesis of homopolyphenylanine in order to compare with 2% cross-linked polystyrene (0.7 mmole/g resin) (Peptide II). On the 1% resin, the reaction proceeds much faster than on 2% resin as shown in Figure 10. 1% resin swells better in various organic solvents. The calculated maximum swelling of cross-linked polystyrene (27) are 49 ml/g and 196 ml/g for 2% and 1% resin, respectively. This clearly demonstrates that 1% resin is superior to the 2% resin. A significantly faster reaction rate is expected since the initial loading of 1% DVB cross-linked resin is also 0.7 mmole/g resin. This reaction rate difference is an evidence for intraparticle diffusion resistance. One disadvantage of 1% resin may be its mechanical instability. A better coupling rate was also obtained in the synthesis of oligodeoxyribonucleotides for 1% DVB resin than for 2% DVB resin (39).

Synthesis of Bradykinin

Bradykinin is one of a group of plasma hormones. It causes dilation of blood vessels and thus a reduction of blood pressure. The amino acid sequence of Bradykinin is Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg. All the Boc amino acids except Gly in Bradykinin have a usable UV-spectrum, but, since the symmetrical anhydride procedure with Boc-Arg(Tos) has been reported to cause double insertion of arginine residue into a peptide and dehydration of the amides, the last attachment of N-terminal Arg was substituted by Pro. In addition, 1% divinylbenzene

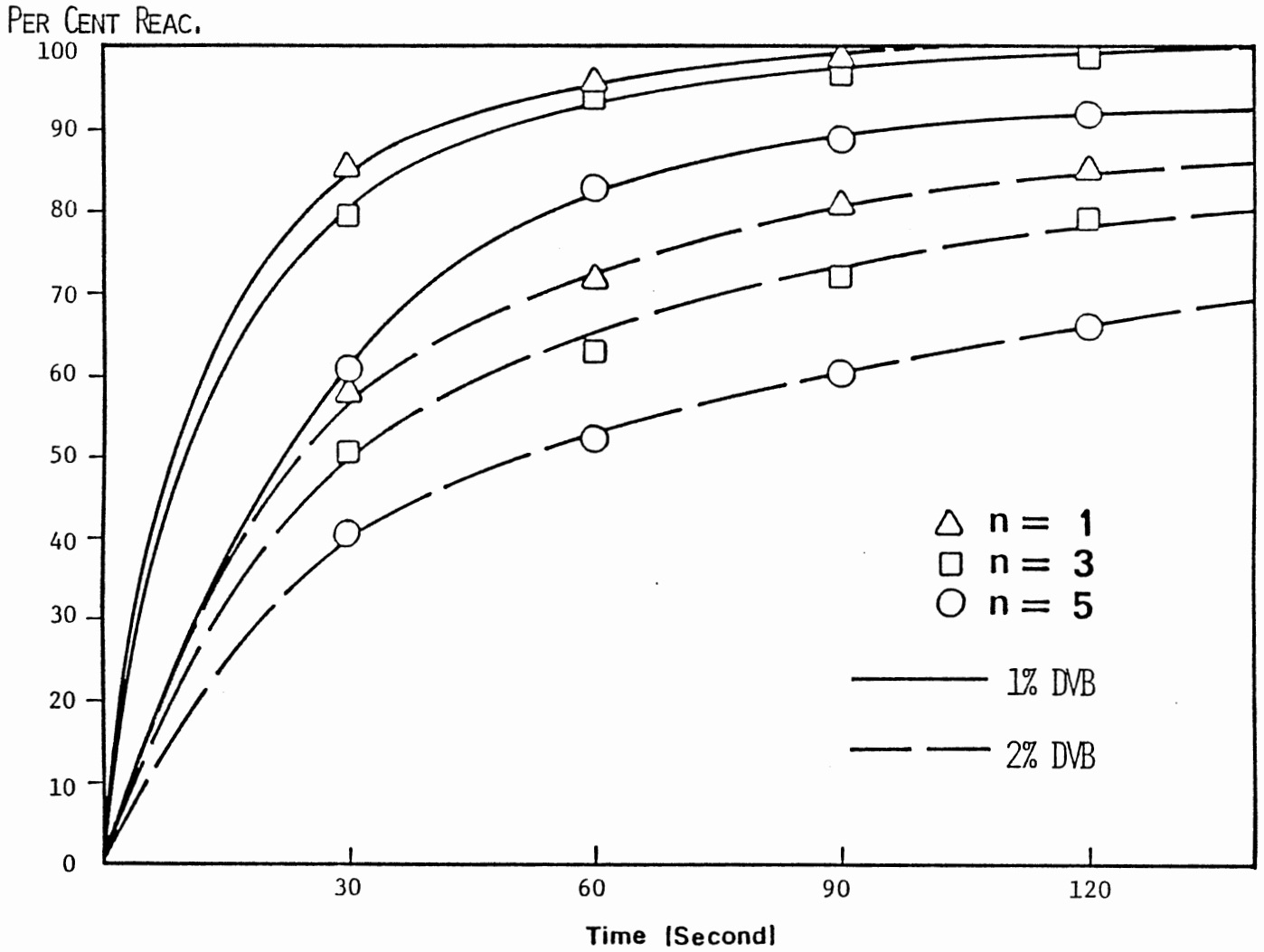


Figure 10. Comparison of Conversion Rate of Amino Terminus on 1% and 2% DVB Cross-linked Polystyrene (Polyphenylalanine)

cross-linked polystyrene with Arg attached (0.45 mmol/g resin) was purchased as starting material.

The purpose of this synthesis is subjected to the applicability of the general trend of results obtained in the synthesis of homopolyamino acids to the synthesis of a biologically active peptide.

Table VIII lists the reaction time versus the conversions.

TABLE VIII

REACTION TIME VS. CONVERSION OF AMINO TERMINUS (BRADYKININ)

Time(sec.)	P-Arg	Phe	Pro	Ser	Phe	Gly*	Pro	Pro
$t_{0.5}$		31	38	18	20	-	17	34
$t_{0.75}$		110	92	36	42	-	39	70
$t_{0.9}$		242	240	66	87	-	61	295

*The attachment of Gly was not monitored. The reaction was completed and tested by picrylsulfonic acid method after 2 hours reaction.

A rapid SPPS of Bradykinin was presented by Corley et al. (40). The technique has two distinct differences from the classical SPPS scheme; First, multiple short periods of coupling or deprotection instead of a single long exposure to a given reagent. Second, insertion of a methanol wash between DCM washes. This development has permitted the synthesis of Bradykinin, in good yield and high purity, in less than five hours. The total time needed for each attachment was given, and

deducting the deprotection, washing, and neutralization time, the coupling reaction for most amino acids was completed in less than 5 minutes. Note that the DCC coupling reaction was the reaction scheme. The actual coupling time and kinetic data were not presented.

CHAPTER VI

DISCUSSIONS

In this chapter, discussions of experimental results will be given and general trends and comments will be presented as they pertain to each of the study objectives given previously.

Experimental Technique Refinement

The first goal of this study was to design the experimental apparatus and to refine an experimental procedure suitable for obtaining ultraviolet monitoring. This final design, along with many of the considerations involved, is outlined in the EQUIPMENT chapter. Several changes were made in the experimental set-up during the progress of experimentation. Two major improvements are discussed here. First, in order to activate protected amino acid, DCC may be added directly to the reactor, however, this results in a DCU precipitation which distorted absorbance readings. To avoid this precipitate inside the reactor, the amino acid symmetrical anhydride procedure was chosen for this experimentation. The anhydride is prepared outside the reactor and the DCU was filtered out before the anhydride solution was mixed with reactive amino terminus of resin in the reactor. In general, couplings with DCC are slower than with anhydrides. This was contributed to the activation process of protected amino acids (23). The second major change was the mixing device, a stream of presaturated nitrogen bubbling through the

reaction mixture used originally for mixing, but a slight amount of DCM was still carried out by nitrogen. This volume change caused the absorbance reading to deviate, especially when the optimum wavelength was not used and when the coupling end point was approaching. Alternatively, mixing was provided by inserting a stirrer through an airtight seal making the reactor a closed system.

Several comments on the formation of amino acid symmetrical anhydride and the initial reading of the UV spectrum are needed. An exact amount of DCC (mole ratio of Amino Acid: DCC=2:1) has to be put in with the amino acid solution to form the symmetrical anhydride at $0^{o}C$ to prevent DCC absorbance which may affect the UV reading from the calibration curve. It should be noted that the UV cut-off wavelength of DCC is about 290 nm which is longer than the working wavelength for all the amino acids in the investigation. The initial UV peak reading for the concentration in the reactor was generally lower than the expected initial absorbance because the anhydride reacts so fast. Reaction mixture pumped out of the reactor at the beginning already reacted to some extent, resulting in a lower initial absorbance reading than the calibration curve. To emphasize the importance of this initial period of reaction, 10-15 sec is needed to circulate the loop of monitoring, by which time 30 percent conversion of serine amino terminus may occur. However, in order to have the right initial absorbance, the anhydride solution prepared was measured directly for each experiment (Experimental Procedure 11). Any effect of absorbance by extra DCC in the reaction mixture could be checked when the total dropping of the UV absorbance curve is as expected from the calibration curve.

Reaction Success, Accuracy and Reproducibility

The second goal of this study was to examine the general success of each reaction and then to determine experimental accuracy and reproducibility. The success of reactions was tested by three techniques. These included a qualitative chemical test, an analysis of the absorbance curves and an overall mass balance for peptide synthesis.

The chemical test, a modified picrylsulfonic acid test (36), was used on every attachment of amino acid. The chemistry background involved is simply that the last amino acid residue has a free amino group while the new residue has its amino group in the form of the N-blocked amide group. A very positive test (little coupling) is indicated by a bright red color to a faint yellow for almost complete coupling (0.001 mmol/g). The resin beads were viewed through a magnifier. This test may be converted to a quantitative version if Beer's law is obeyed. Anyway, a more quantitative test, such as the quantitative ninhydrin procedure (15), should probably be used for completeness and thoroughness. However, a complete coupling has to be observed by picrylsulfonic acid method before proceeding to another attachment. In terms of reproducibility, the synthesis of Peptide I and II are replications in fairly good agreement (Appendix C).

Determinate errors that are associated with this experimental set-up basically include the error caused by the monitoring loop volume, mixing, the volume change and the convection dispersion in the monitoring loop. Reaction mixture volume changes have been eliminated by this closed system design and convection dispersion in the monitoring loop is negligible since a small bore teflon tubing (0.095 in ID) was used. The error caused by the volume of the monitoring loop was

calculated by a discrete method to approximate this continuous process. Less than three percent error was obtained for the first 30 seconds of reaction at residue number 3 for synthesizing polyphenylalanine (Peptide II). The error percentage should go down as reaction proceeds. Sample calculations are in Appendix B.

Data Analysis

The analysis of the absorbance curves in order to have reaction rate information is the major feature of this investigation, and this analysis involves comparing the total drop in absorbance to the drop predicted by the calibration curves, and the rate of dropping.

The UV-absorbance difference between the initial reading and the end point of coupling is regarded as the total dropping. When this compares with the predicted value from the calibration curve in good agreement, the amino terminus is assumed completely converted. But the accuracy is limited partially because the optimum wavelength has not been used, this causes a large absorbance value change only by a slight volume change of reagent or the wavelength inaccuracy (± 0.5 nm, Perkin Elmer Lambda 3), and partially because of the amount of DCC used in the symmetrical anhydride formation as discussed in Experimental Technique Refinement. However, one hundred percent conversion of amino terminus is assigned to the measured total absorbance dropping whenever the negative picrylsulfonic acid test is obtained and the total dropping is in good agreement with predicted value. Examples of absorbance curves for the synthesis of Peptide II (polyphenylalanine, n = 3 and n = 5) were traced directly from the recorder, and shown in Figures 11 and 12. Those two figures illustrated both the initial absorbance reading

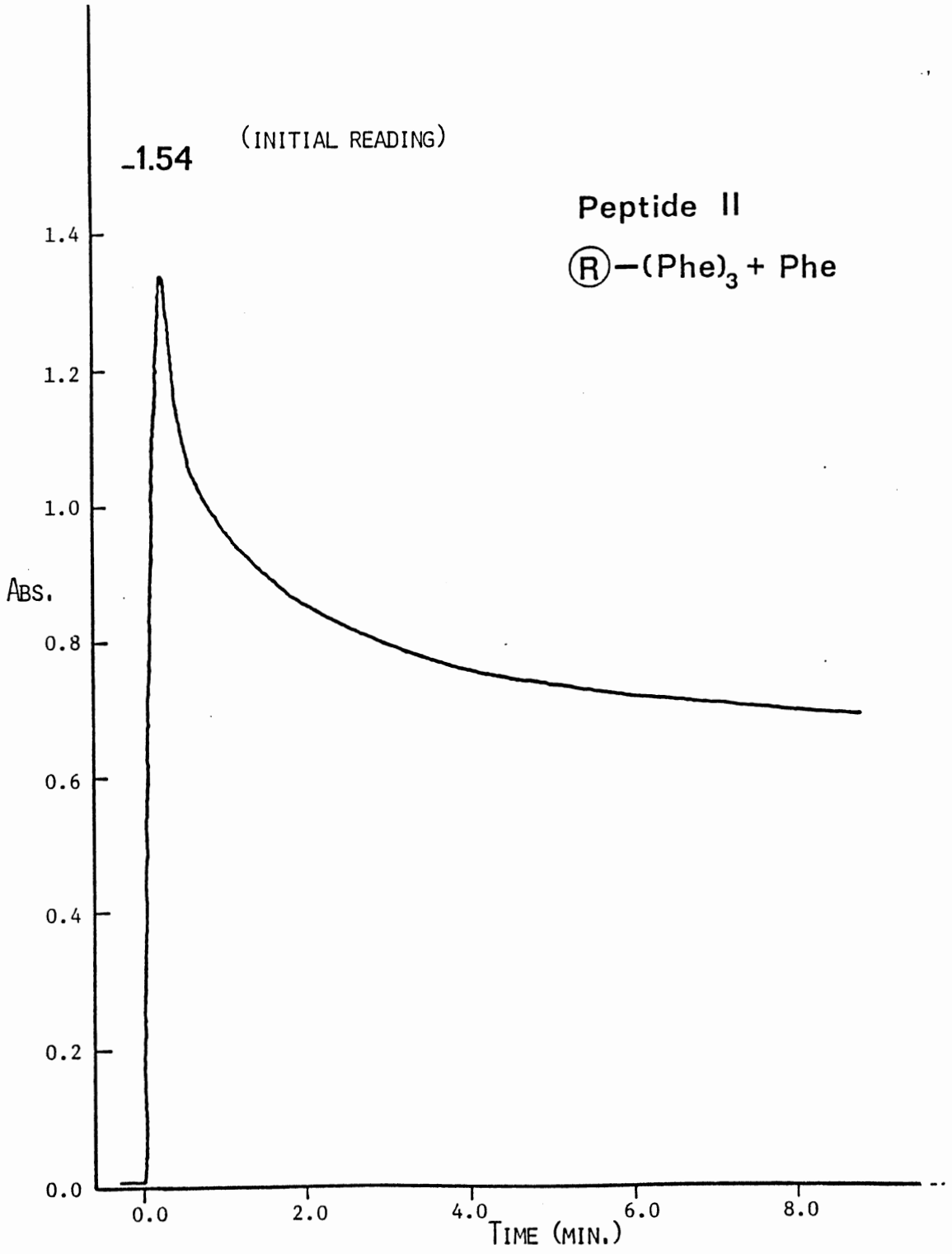


Figure 11. Absorbance Curve of Phe Attachment to Resin-$(Phe)_3$ in Peptide II (Phe)

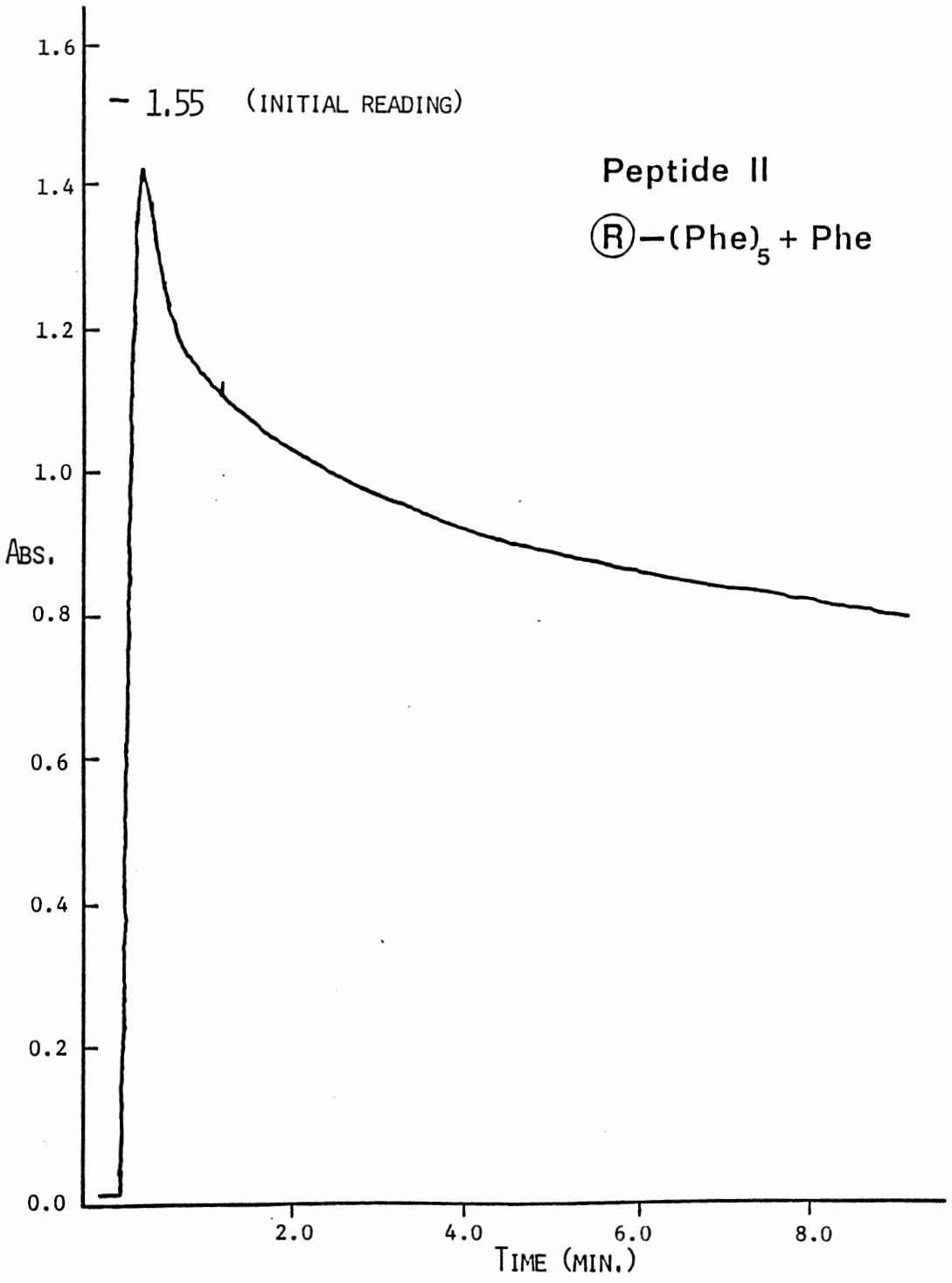


Figure 12. Absorbance Curve of Phe Attachment to Resin-$(Phe)_5$ in Peptide II (Phe)

(Experimental Procedure 11) and the rate of absorbance change. In these two attachments, the measured total dropping of absorbance were 0.873, while the calculated value from the calibration curve is 0.868, the difference is within the standard error of absorbance estimated. Then, the kinetic data can be obtained from the absorbance curves by the following procedures. First, the initial period's (about first 20 sec.) data is extrapolated from the absorbance curve to the initial reading. Second, the absorbance readings are taken carefully at every time interval and transferred to concentration of anhydride and, at the same time, substituted into any tried kinetic expression. In Dietrich's thesis (37), $\ln (-dC_A/dt)$ is plotted against $\ln (C_A)$, where C_A is the concentration of amino terminus, to give a graph with slope equal to the apparent reaction order. The problems and limitations caused by this analysis were well explained by Dietrich.

Since a low excess ratio of symmetrical anhydride was used in this study, it is reasonable to apply the ideal second order reaction rate to fit the process of coupling reaction.

$$- \frac{dC_A}{dt} = k_2 (C_x) (C_A) \qquad (2)$$

where C_A is the concentration of active sites on the resin or the amino terminus of attached peptide and C_x is the concentration of anhydride, and $(C_x - C_A)$ is a constant in this case. The results of Peptide II(Phe) synthesis were conducted to discuss the applicability of Equation 2. Merrifield (23) and Rudinger (20), respectively, have applied second order kinetics to express some of their rate measurements in solid phase synthesis. Comparisons and discussions between these studies follow.

In Merrifield's paper, second order reaction rate constants were listed for coupling between Bpoc-amino acid anhydride with ala-resin and val-resin, and it has been found that ideal kinetics are observed to at least 90% completion. This finding of ideal kinetics was not reported as the number of residues increased. The general observation of this study shows that the deviation from ideal second order kinetics increases as percent conversion of amino terminus and the number of residue in peptide increases. Furthermore, for example, a reported rate constant for the coupling of Bpoc-Leu with Ala-resin is 6.35 l/mol sec implies that the 99% reaction can be therefore estimated to be 14 sec. Such a fast reaction rate brings in a lot of questions about how their experimentation was done and how the data has been analyzed.

On the other hand, Rudinger reported that the "apparent" second order rate constant, k_2' decreases as percent of conversion of amino terminus increases. The apparent rate constant is the multiplication of true rate constant by the factors contributed by resin swelling and partitioning of reagents between bulk solvent phase and solid phase. It should be noted that Boc-protected amino acid active esters react with aminoacyl or peptidyl resins in the synthesis scheme in Rudinger's study, and the reaction rates are about 10 fold slower than the Bpoc-symmetrical anhydride scheme. For both papers, the attachment of amino acid to aminoacyl resin satisfy the second order rate expression, but when the number of amino acid residues in the peptide increase, deviation from ideal second order kinetics occurs. However, it has to be cleared that the mole ratio between initial amino acid and active site of resin was not reported in Merrifield's paper and was between 1.2 and 3 in Rudinger's experiments.

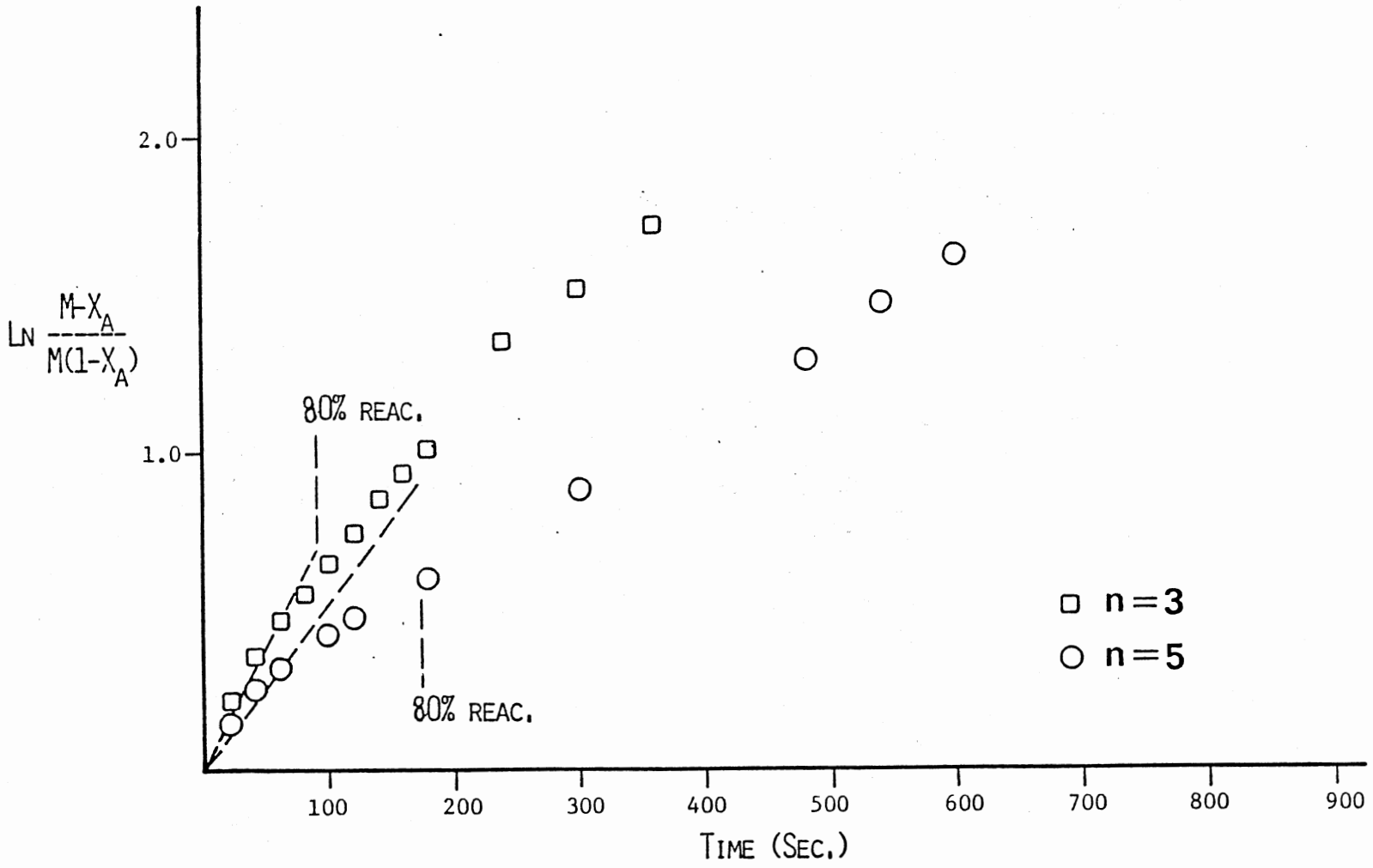


Figure 13. Second Order Plot of $\ln((M-X_A)/M(1-X_A))$ Versus Time in the Attachment of Phe to Resin-$(Phe)_3$ and Resin-$(Phe)_5$ in Peptide II (Phe)

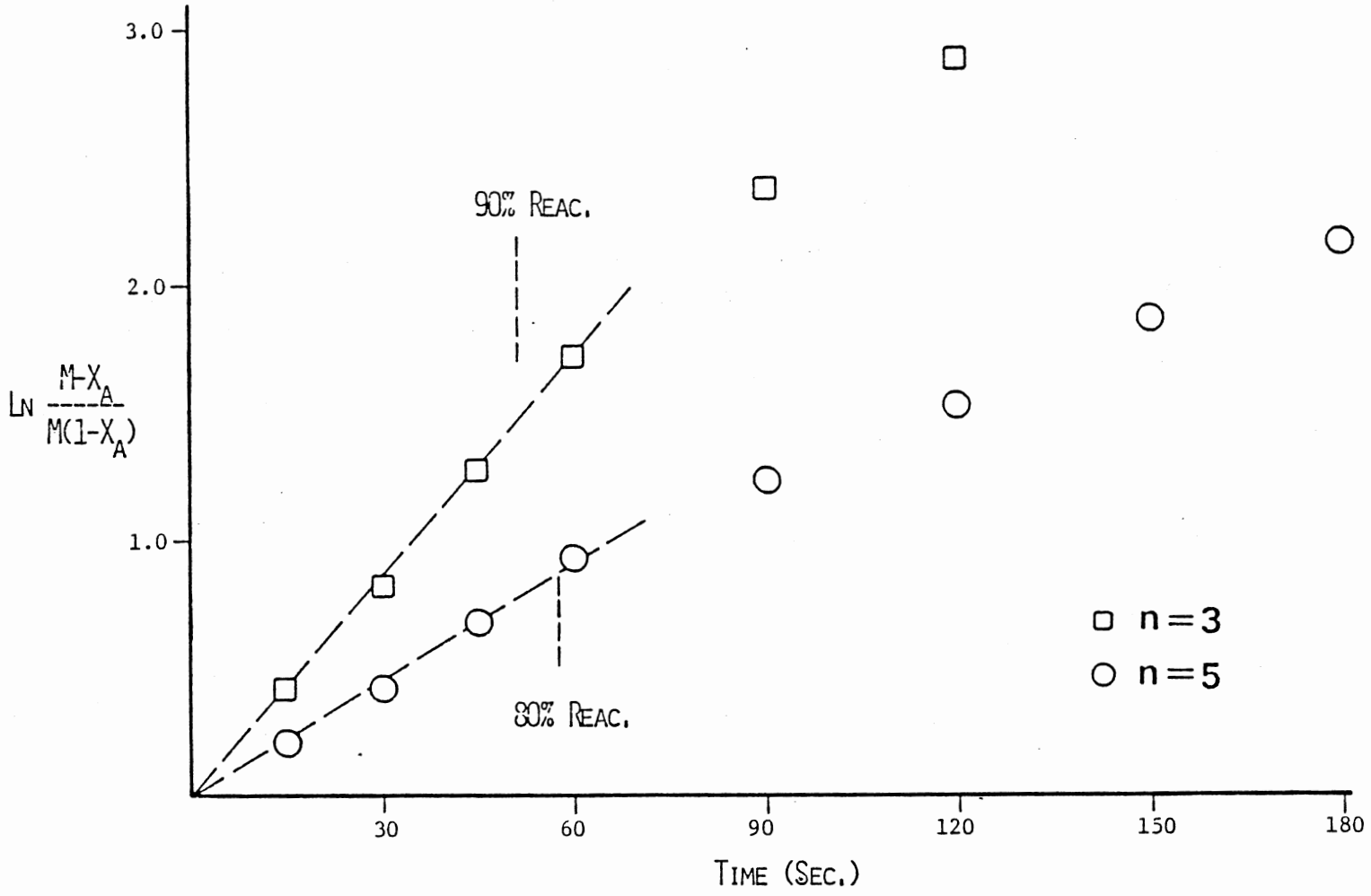


Figure 14. Second Order Plot of $\ln((M-X_A)/M(1-X_A))$ Versus Time in the Attachment of Phe to Resin-$(Phe)_3$ and Resin-$(Phe)_5$ in Peptide VII

Second order kinetics was forced to fit to the synthesis of Peptide II (Phe) and Peptide VII (Phe), and the rate constants were generated. Table VII is a list of second order rate constants, as in Equation 2, based on the initial 50 percent conversion of amino terminus.

Sample graphs of $\ln((M-X_A)/M(1-X_A))$ versus time are presented in Figures 13 and 14 for Peptide II (Phe) and Peptide VII (Phe) respectively. As shown in these figures, the slope which is the ideal second order rate constant k_2 times $((C_{xo}-C_{Ao}))$ decreases as the conversion of amino terminus or the reaction time increases. Also, comparison of two resins based on copolymers with 2% and 1% DVB, in Figure 14 shows that the rate of reaction of 1% DVB resin is not only higher but also constant over a wider measured range, once more confirming the superiority of resin with low cross-linking.

TABLE IX

SECOND ORDER RATE CONSTANTS OF PEPTIDE II(PHE)

Boc-amino acid	Resin-(Phe)+Phe	k_2(l/mole sec)*
Phe	1	5.61×10^{-1}
Phe	3	4.87×10^{-1}
Phe	5	3.39×10^{-1}
Phe	7	1.85×10^{-1}

*See Appendix D for sample calculations

Reaction Parameter

It has thus far not been possible to establish optimum reaction times and other parameters for SPPS. The attempts of this investigation are basically preliminary analysis of effect of the reaction parameters on SPPS. The knowledge of these parameters is essential for the optimization especially since there are so many reaction variables involved in this heterogeneous and amino acid sequency dependent reaction. The reaction parameters studied in this thesis include chain length of peptide, mixing rate, reaction temperature, excess mole ratio between amino terminus and symmetrical anhydride, and the polymer support.

Peptide Chain length

The coupling rate decreases as the chain length increases in the synthesis of homopolyserine and homopolyphenylalanine but not in the synthesis of Bradykinin. Generally the chain length increase may increase the electron donating ability of the amino terminus, pKa, which, in turn increases the reactivity of amino terminus of nucleophilic attack by carboxyl group. This phenomenon of the pKa value increase on SPPS of a specific peptide (Gly-Asn-Thr-Ile-Val-Ala-Val-Glu) was reported by Hooper (31). The author determined the pKa value by a titration method but, unfortunately, did not report any amino terminus convertion rate. The side chain of each amino acid has the ability of affecting electron transfer, and the impact of secondary structure of peptide has also to be considered on peptide chain length effect on coupling rate.

According to Sarin's study, there is no indication that the resin matrix becomes filled with peptide as it grows, nor does the efficiency of the synthesis become limited by an overcrowding or steric effect (27). Thus, the steric hindrance of an individual amino acid may be caused by a difficult orientation match with the peptide amino terminus but not because of diffusion limitation in the polymer network. In the synthesis of Bradykinin, a longer coupling half-time for Pro (residue 8) was observed than for Pro's (residue 7), but shorter than Pro at n = 2 since Pro (n=2) is attached to a different amino acid, Phe, which has known lower reactivity compared with Gly. Thus, it appears that a chain length effect exists and is based on the following two general observations in this study. First, the coupling rate between amino acids depends on both amino acid's reactivity, for instance, the high reactivity of Ser result in faster reaction rate of Phe (n = 4) than Phe (n = 1), and, secondly, for the same reactivity, the coupling rate decreases as peptide chain grows. However, the secondary conformation of homopolyamino acid, the tendency for random formation, may influence the microphysical environment of accessibility to the amino terminus. A significant decrease in coupling at n = 4 for homopolyamino acids may be associated with the accessibility of this oligomer. The accessibility of the amino terminus is determined by its attached peptide and the solubility of the peptide in solution, which in turn, relates to the peptide's hydrophilic or hydrophobic property and the reaction solvent.

A good illustration of the solubility problem can be found in the synthesis of homopolyisoleucine by different methods (23). In the solution method, it is quite difficult because the growing peptide become insoluble in the reaction medium, DCM. After n = 4 the peptide

chain could not be extended as a result of this insolubility. But, on the solid phase method, poly(Ile)$_{12}$ could be readily assembled by symmetrical anhydride couplings in DCM. In this case, rather than being the cause of such sequence-dependent problems, the polymer support (1%-divinylbenzene cross-linked polystyrene) actually helps reduce the difficulties encountered with peptides that tend to self-aggregate. Thus, such information about the role resin plays in the conformation of a growing peptide could be helpful to explain the reaction rate.

Reaction Temperature

The experimental set-up is capable of running reaction temperatures lower than room temperature. In practice, most of the experiments of SPPS are carried out at 25^{o}C in the methylene chloride reaction medium. Due to the high volatility of DCM (B.P. = 39.8^{o}C) a lower temperature, 14^{o}C, was used to repeat the synthesis of Peptide I and II.

The same chain length effects were observed in the synthesis of Peptide III and IV at the lower temperature. A sharp increase in reaction time occurs at residue number 4 at lower reaction temperature. This phenomena is true for both serine and phenylalanine and for other reaction parameter changes, like mole ratio. However, this dependency could not be quantified based on only two temperatures. Table X shows the trend that lower temperature decreases the coupling rate with the effect enhanced as the peptide chain grows, however, the numbers presented do not conform to any predictable formula. The enhancement of coupling rate at elevated temperature provides indirect evidence for the microphysical nature of coupling difficulties, such as the freedom of peptide chain vibration and physical aggregation due to hydrogen

bonding, and the high reaction temperature might be an useful solution to overcome some low reaction rate problems.

Based on an ideal second order reaction rate approximation, the apparent activation energy for the synthesis of polyphenylalanine at residue number 3 is 2.9 Kcal/gmole (see Appendix D) which indicates a temperature insensitive mechanism, intraparticle diffusion, may be the reaction control step. However, a sharp increase in reaction time at a latter stage of coupling and attachments imply a higher value of activation energy which may be attributed to the collision rate decreases caused by secondary structure or by the reaction control mechanism change. This is still unclear.

TABLE X

THE EXTENT OF TEMPERATURE EFFECT IN THE SYNTHESIS OF PEPTIDE I (SER) AND III (SER)

	Reaction Time(sec)	Conversion %		% Reduced
		Peptide I ($26^{o}C$)	Peptide III ($14^{o}C$)	
n=3	T=120	89.8	85.5	5.0
	T=240	97.9	92.5	3.8
	T=360	99+	96.5	2.7
n=5	T=120	85.0	76.0	11.0
	T=240	94.3	87.1	8.0
	T=360	97.7	93.0	5.0

It is worth mentioning that Tam (35) has promoted the reaction rate and coupling efficiency on a peptide for difficult synthesis sequences of amino acid by changing DCM to 1-methyl-2-pyrrolidinone and increasing the reaction temperature to 122 F.

Mole Ratio

The excess mole ratios of 1.5 and 1.2, symmetrical anhydride to amino terminus, were performed on polyphenylalanine synthesis (Peptide II and Peptide V). There are reasons to believe that the excess of Boc-amino acid can be regained after the reaction is complete. However, these factors considered in economic aspect are beyond the scope of this study. The excess carboxyl groups do increase the coupling rate between amino acids, which indicates that the reaction step does not dominate the whole reaction process. In other words, the diffusion process of reagent, including film diffusion and particle diffusion, might be significant in reaction rate for this heterogeneous reaction, especially at the latter stage of synthesis of polyphenylalanine at $n \geq 4$. The film diffusion resistance has been ruled out in the range of mixing rate under investigation in this study. Two experimental results may help understanding of diffusion in the polymer network. First, according to Merrifield's study (23), the swollen space of peptide-resin available for peptide chain growth within the resins increase during repeated reactions rather than being gradually filled by peptide. Secondly, the NMR study done by Kent and his coworkers (29), in DCM and DMF, the interior of the 1% cross-linked polystyrene network is a homogeneous environment of well solvated highly mobile chains with properties analogous to a solution of linear polystyrene. The above results may

lead to a conclusion that particle diffusion resistance is not a rate limiting step.

Thus, the increase mole ratio phenomena could not be explained by a simple heterogeneous reaction model, and this increase coupling rate by increasing mole ratio is related to the chain length of peptide suggesting that the peptide chain may introduce an orientational difficulty of collision between reagents. The tendency of coil formation of homopolyamino acid (41) results in reduced accessibility of the amino terminus.

Kinetic Model

The knowledge of the reaction mechanism is limited. The deviation from ideal reaction kinetic has been explained by the heterogeneous reactivity of reactive site within the polymer resin (32, 42). This is still not positively confirmed. In addition, the general observations in this study that the peptide chain increase in the polyamino acid synthesis could not be well explained by the heterogeneous reactivity. Besides, the physico-chemical properties of resin-bond peptide studied by Kent and coworkers (29) show that all sites in the resin network are readily accessible and there will not be a dispersion in reactivities. So, basically, this controversy is still going on.

One of the purposes of this study is to develop a reaction mechanism and a kinetic model. Previously, Dietrich attempted to find the reaction order and rate constants by plotting ln $(-dC_A/dt)$ versus ln (C_A), a line with slope equal to reaction order and y-axis intercept equal to ln (rate constant). This type of graph is particularly useful in determining shifts in reaction order. A sample of Dietrich's graph

is presented in Figure 15. Several conclusions can be made by these results. First, apparent zero and first order reactions occur in the early parts of the reaction, while second order reactions occur in the latter stages with lower symmetrical anhydride concentration. Also, as chain length increases, the jump to higher orders, with respect to sites converted, occurs earlier in the reaction.

In this study, for low excess mole ratio between symmetrical anhydride and reactive site on the resin, a second order reaction rate expression has failed to fit the data (see Data Analysis in this chapter and Figures 13 and 14). The following reaction mechanism and reaction rate expression are simply hypothetical and based on the experimental results obtained in this study.

Before a model is proposed, a couple of facts about the physicochemical properties and the experimental results that can be used to support the proposal have to be stated. First at all, an autoradiograph study (23) shows a uniform distribution of polymer backbone and peptide chains of polystyrene resin in the polymer network. The movements of fully stretch of the peptide chain length only occupies less than 5% of the void space of resin in the reaction medium (DCM or DMF). This implies that the diffusion process inside a particle may not be a rate determining step. This is supported by Merrifield's calculation that diffusion rate is 10 times faster than reaction rate (23). However, in this study, higher excess mole ratio (1.5) did significantly change the coupling rate for the number of residue larger than 4, which indicates that the diffusion step is dominating the whole reaction process. Since the chain length increase does not physically block any of the void space in the resin for

particle diffusion, the peptide chain itself may introduce a reduction of amino terminus accessibility. The only explanation is the conformation of the peptide chain affects the accessibility of the amino terminus, this orientation problem is related to the peptide's characteristic in the reaction medium, and this only appears when the conformation of peptide has steric hindrance for the collisions between active sites of amino terminus and anhydride. This is reasonable since different reaction medium or different polymer support have reduced the difficulty associated with sequencing amino acids.

However, a simple shifting order kinetic model can take some of these observations into account, that is

$$- r_A = \frac{K_1 C_A}{K_2 + C_A} \tag{3}$$

where $-r_A$ is the rate of disappearance of reactive site on the resin, and K_1, K_2 are rate constants. Several mechanisms can result in this empirical rate form, but, basically, a reaction intermediate has to be formed. After rearrangement, a plot of $X_A C_{Ao}/\ln(1/(1-X_A))$ versus $t/\ln(1/(1-X_A))$, where t is reaction time, gives an intercept, K_2 and slope, $-K_1$. This plot works well up to 95% conversion of reaction sites through residue 8 for both phenylalanine and serine, but higher deviations were observed at a higher residue number. Figure 16 is the plot for peptides I (Ser) and III (Ser), showing the different reaction temperatures. Figure 17 is the result for peptide II (Phe) and V (Phe) for different excess mole ratios. Figure 18 compares polymer supports (1% and 2% DVB polystyrene). Above 95% conversion the plotted values scatter badly as X_A approaches 1; these calculated points are not shown.

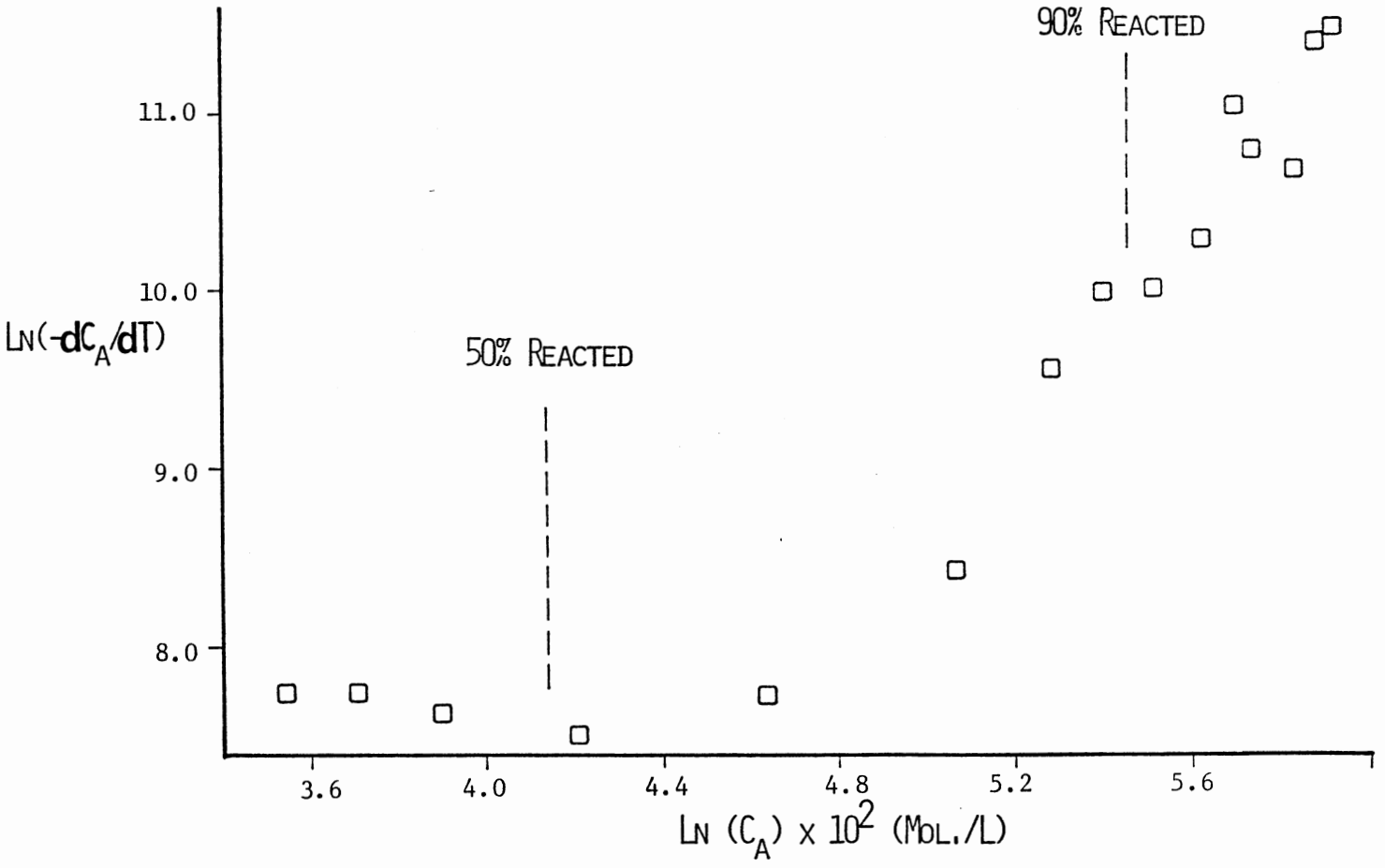


Figure 15. Apparent Order Plot for the Attachment of Phe to Resin-Pro-$(Phe)_3$

Table XI gives the K_1 and K_2 value for the peptides synthesized. Note that K_2 is an optimized value that makes the K_1 fit all straight lines in Figures 16, 17, 18, and has the least difference with experimental results. No direct comparison could be found with published data because of different kinetic schemes and reaction processes. However, general conclusions can be made. First, this kinetic model, Equation 3, has a better fit, especially at the latter stage of attachment, than the ideal second order reaction rate expression. However, for the latter stage of peptide synthesis, more detailed understanding about the reaction microenvironment is needed in order to set up a better reaction mechanism and reaction model. Second, K_1 is a function of amino acid, peptide chain length, reaction temperature and excess mole ratio. K_1 decreases as chain length increases, while K_2 remains constant. Also, K_2 may be constant for any amino acid in this study since the degree of substitution (loading of resin) varies for Phe (0.7 mmol/g) and Ser (0.2 mmol/g). For peptide I, K_2 = 0.008, multiply by 0.7/0.2 to give 0.028; for peptide III K_2 = 0.0075, multiply by 0.7/0.2 to give 0.026; as a result, all values of K_2 are approximately the same for different initial amino acid loading of resin, reaction temperatures and numbers of amino acid residue in peptidyl-resin. Although there are limitations of applicability of Equation 3, the constant value of K_2 helps simplify reaction predictions and reactor modeling.

For K_1, which is a function of chain length, the negative slope decreases with increasing n. As a result, a more specific form of Equation 2 may be appropriate.

$$- r_A = \frac{k_1 C_A}{n(k_2 F + C_A)} \tag{4}$$

where n is the number of residues on the existing chain and F is a degree of substitution factor for the acyl resin. Substantially more experimentation is required to verify and/or quantify this hypothesis.

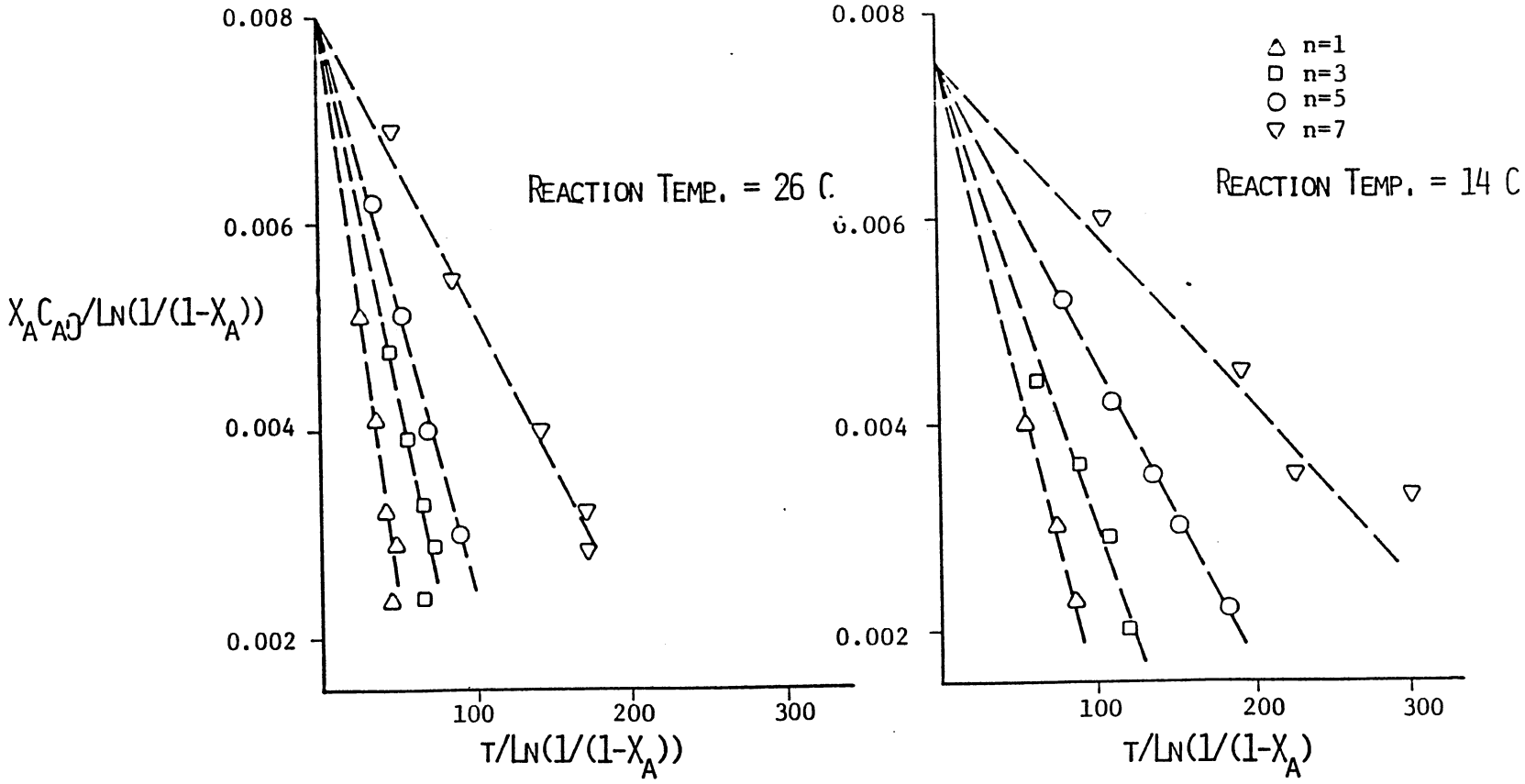


Figure 16. Shifting Zero to First Order Plot of $X_A C_{AO}/\ln(1/(1-X_A))$ Versus $t/\ln(1/(1-X_A))$ of Peptide I (ser) and Peptide III (Ser) with Respect to Reaction Sites

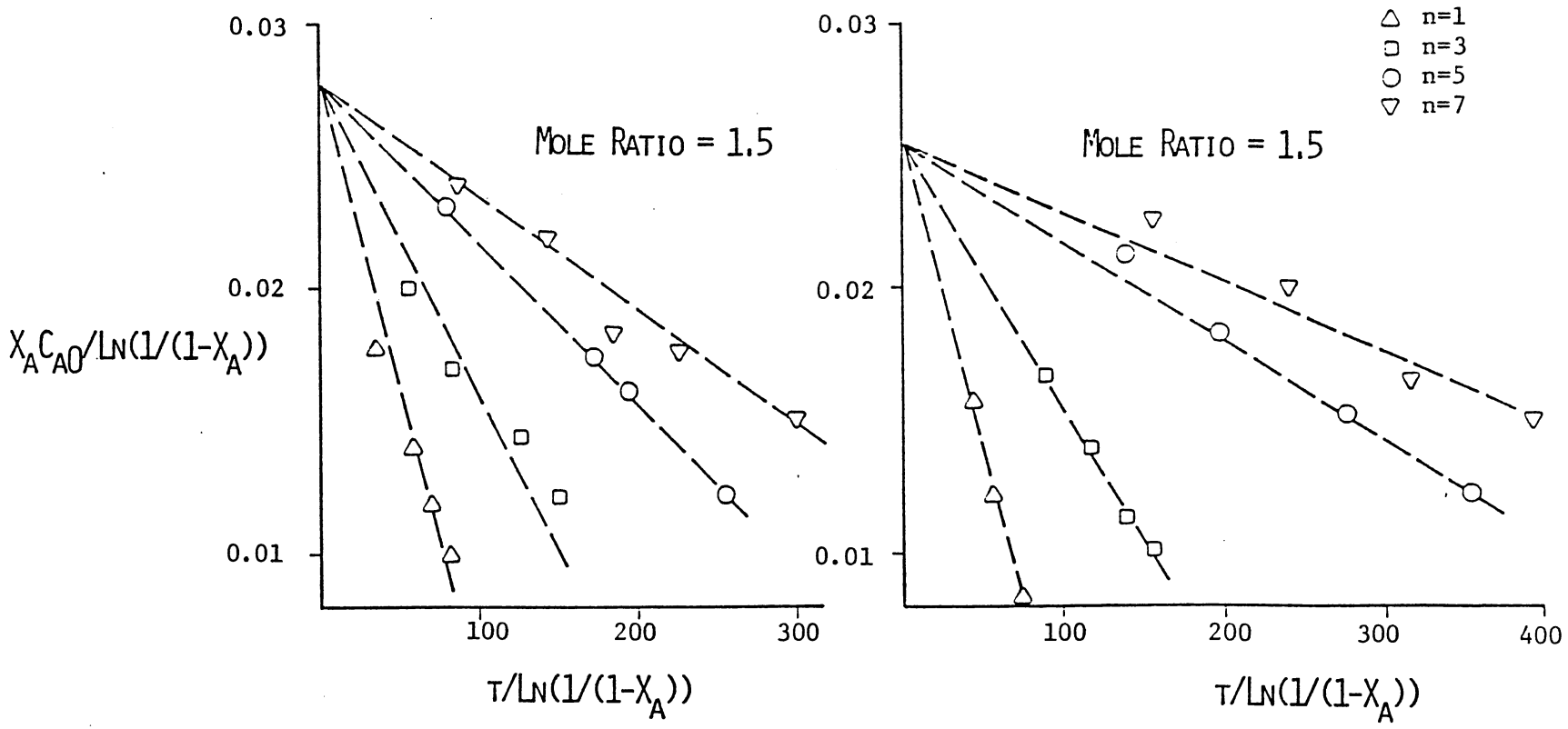


Figure 17. Shifting Zero to First Order Plot of $X_A C_{A0}/\ln(1/(1-X_A))$ Versus $t/\ln(1/(1-X_A))$ of Peptide II (Phe) nad Peptide V (Phe) with Respect to Reaction Sites

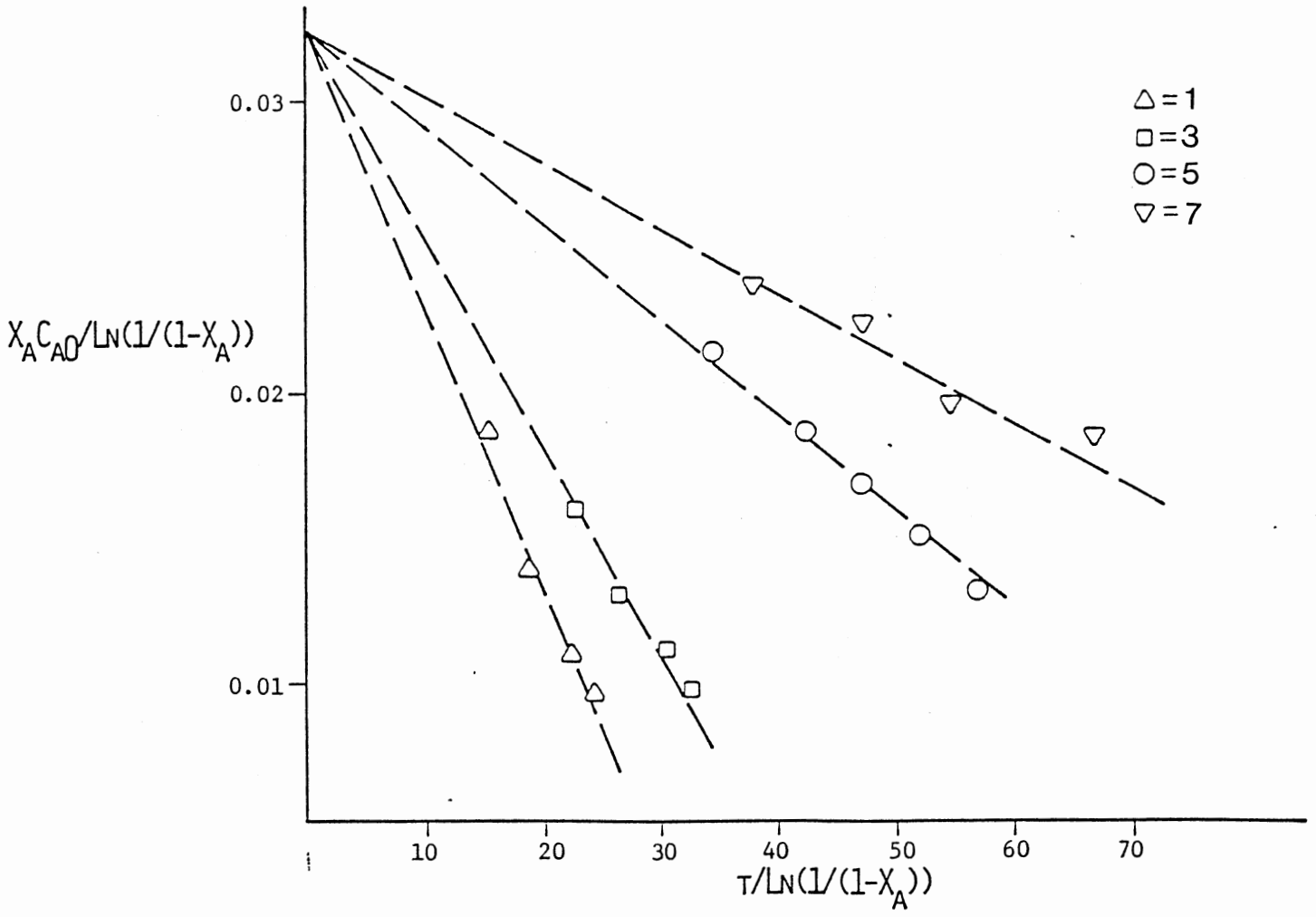


Figure 18. Shifting Zero to First Order Plot of $X_A C_{A0}/\ln(1/(1-X_A))$ Versus $t/\ln(1/(1-X_A))$ of Peptide VII (Phe) with Respect to Reaction Sites

TABLE XI

REACTION RATE CONSTANTS K_1 and K_2 FOR THE SYNTHESIS OF POLYPHENYLALANINE AND POLYSERINE BASED ON THE PROPOSED EMPIRICAL RATE EQUATION

Peptide	n	k_1^a(mmol/ml.sec)	k_2^a(mmol/ml)
Peptide I (Ser)	1	1.28×10^{-4}	8.0×10^{-3}
	2	1.22×10^{-4}	"
	3	8.88×10^{-5}	"
	4	7.19×10^{-5}	"
	5	6.46×10^{-5}	"
	6	5.38×10^{-5}	"
	7	3.26×10^{-5}	"
Peptide III (Ser)	1	7.32×10^{-5}	7.5×10^{-3}
	2	7.02×10^{-5}	"
	3	5.17×10^{-5}	"
	4	3.87×10^{-5}	"
	5	3.43×10^{-5}	"
	6	2.81×10^{-7}	"
	7	1.97×10^{-5}	"
Peptide II (Phe)	1	3.17×10^{-4}	2.78×10^{-2}
	2	2.97×10^{-4}	"
	3	1.56×10^{-4}	"
	4	8.54×10^{-5}	"
	5	7.94×10^{-5}	"

TABLE XI (Continued)

Peptide	N	k_1^a(mmol/ml.sec)	k_2^a(mmol/ml)
	6	6.89×10^{-5}	"
	7	5.79×10^{-5}	"
Peptide V (Phe)	1	3.27×10^{-4}	2.54×10^{-2}
	2	2.55×10^{-4}	"
	3	1.43×10^{-4}	"
	4	6.28×10^{-5}	"
	5	4.95×10^{-5}	"
	6	3.42×10^{-5}	"
	7	2.68×10^{-5}	"
Peptide VII (Phe)	1	0.44×10^{-4}	3.16×10^{-2}
	2	8.54×10^{-4}	"
	3	7.01×10^{-4}	"
	4	4.12×10^{-4}	"
	5	3.28×10^{-4}	"
	6	2.73×10^{-4}	"
	7	2.18×10^{-4}	"

[a]See Appendix D for sample calculation

CHAPTER VII

CONCLUSIONS AND RECOMMENDATIONS

In light of the experimental results and development efforts of this research study, the following conclusions can be drawn:

1. Continuous monitoring of the absorbance of the liquid phase during peptide synthesis is a convenient way of measuring the coupling reaction rates in a solid phase system. Less than 3 percent determinate error was calculated based on discrete approximation for the initial stage of coupling at n = 3, and the error should go down as reaction proceeds. These measurements can yield information about the reaction parameters and assist in the rational choice of materials and conditions for better practical process result. The procedure in its present form is not adequate for determining reaction end points since the reaction approaches completion asymptotically.

2. With the assistance of the qualitative picrylsulfonic acid method, the end point of reaction and the percent conversion of amino terminus as a function of time can be determined. This can be utilized to optimize the reaction time for the peptide synthesis process. But, based on the literature survey, for synthesizing various peptides, a routine process may not be enough to optimize production. Changing the reaction medium, protection group of amino acid, and the reaction parameters ... etc, could only result in the best performance.

3. The coupling rate between the amino terminus and symmetrical anhydride of amino acid decreases with increasing chain length, or the number of residues in the peptide, increases. This chainlength effect is true also for a lower reaction temperature ($14^{o}C$), different excess mole ratio, and mixing rate. The change of mixing rate from 100 RPM to 200 RPM does not affect the coupling rate, indicating that film diffusion resistance of this heterogeneous reaction is not a rate determining step in this study. The lower temperature reduces the rate of reaction and the excess carboxylic group elevates the coupling rate between amino acids. These general observations provide a possible explanation of this heterogeneous reaction mechanism, that is, the amino acids or amino acid derivatives can flow freely into or out of the resin phase. The film and particle resistance do not exist in this case, and the reactivity of the amino terminus is a function of chain length which is induced by the conformation of peptide.

4. The mechanism of the coupling reaction is still unclear, but an empirical reaction rate expression ($-dC_A/dt = K_1C_A/(1+K_2C_A)$) can be based on this experimental results. Empirically this is a order shifting reaction, the rate constant, K_1, is a function of temperature, excess mole ratio, polymer support and amino acids, and K_2 is only a function of amino acid and polymer support.

The above results are based on the synthesis of short peptides (homopolyphenylalanine and homopolyserine) in various reaction condition. More importantly, several assumptions were made about the experimentation and data analysis which are still vague. Some recommendations learned from this study may help clarify for future studies.

1. The liquid phase change is assumed to exactly correspond to the change in the solid phase. This includes both absorption and carboxyl-amino groups interaction. Both reduce the concentration in the liquid phase and are interpreted here as reaction consumption. According to Ragnarsson et al. (24, 25), dioxane, which has basic properties and is not inert to proton donors, could be used as a reaction medium to eliminate the possibility of ion-pair formation.

2. A portion of liquid phase (bulk phase) was pumped through the circulation loop in order to obtain absorbance data. This circulation loop can be counted as dead volume in the reactor. This dead volume affects not only the concentration of active sites of resin but also the anhydride and creates a delay in reaction data acquisition, and, if convection dispersion does happen, could result in data inaccuracy. Thus, reduction of the circulation loop volume is desired. This can be partially done by reducing the flow cell capacity, the size of the three-way stopcock, and the length of Teflon circulation tube.

3. Adjusting the optical path of the flow cell an optimum wavelength of UV-spectrum may help in data reproducibility and accuracy.

4. Amino acid analysis is needed for future assessment of any amino acid sequence. This was only done, in this study, by a material balance of the resin before attachment, and the resin and peptide after the experiment.

5. A more detailed kinetic study based on this experimental set-up can be done on the following kinetic variables; First, the resin particle size, the relationship between particle diameter and the rate of conversion can give insight into the rate determining step for this

heterogeneous reaction process. Second, reaction solvent volume - concentration of resin active sites and amino acid or amino acid derivatives may change the role of mass transfer in the reaction process for various peptides. In addition, the partition coefficient and rate of equilibrium between the bulk phase and the reaction phase inside the resin are also needed.

6. The relationship between secondary structure of peptide attached to the resin and the accessibility and activity of amino terminus is highly desirable. For instance, the change from random coil to -helix may be accompanied by a drastic change of peptide solubility and in turn, may decrease the reactivity of the amino terminal. In addition, pH and temperature may affect the structure of homopolyamino acid, this investigation may be accomplished by the supporting information from CD (circular dichroism) or NMR.

7. Autoradiograph study of peptide-resin during the coupling reaction is helpful in understanding the reaction model.

In summary, the results presented in this thesis are based on the model peptides under investigation. Interrelation between secondary conformation and physical-chemical properties of a specific peptide were not brought into the synthesis of these model peptides. Different results may be observed for other system. However, this study provides an initial basis for further kinetic study of SPPS.

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APPENDIX A

LISTS OF CALIBRATION CURVES FOR AMINO ACIDS AND ANHYDRIDES

TABLE XII

CALIBRATION CURVE EQUATIONS

Amino Acid	Wavelength (λ)	Absorbance[a]	Standard Error Estimated
Phe	274	0.0068+9.7436C	0.0440
	276	0.0062+5.8087C	0.0
Phe Anhydride	274	0.0078+49.953C	0.0069
	276	0.0814+31.483C	0.0798
Ser	272	0.0537+18.837C	0.0159
	274	0.0199+9.277C	0.0067
	276	0.0090+4.350C	0.0020
Ser Anhydride	272	-0.0143+70.770C	0.0193
	274	-0.0263+47.401C	0.0112
	276	-0.0338+34.614C	0.0103

a. C is concentration of either the amino acid or anhydride. Concentration ranges between $4X10^{-3}$ to $7X10^{-2}$ mmole/ml DCM.

APPENDIX B

ERROR ANALYSIS FOR CIRCULATION LOOP

APPENDIX B

ERROR ALALYSIS FOR CIRCULATION LOOP

In order to describe the indeterminate error caused bt the circulation loop, a discrete approximation was chosen and perfect mixing in the was assumed. The following information are necessary for this error analysis.

The reaction mixture volume = 30 ml
The circulation loop volume = 3 ml
The time needed for circulation = 10 sec.
Time interval for analysis = 10 sec.

Time (sec.)	Absorbance (λ= 276)	% reacted
0	1.540	
20	1.186	33
40	1.035	47
60	0.978	53
80	0.930	57
120	0.865	61

At first time interval (0-10 sec.)

Take 3 ml liquid phase of reagent mixture at T=5 sec., and the UV-absorbance is 1.4515 approximately.

At second time interval (10-20 sec.)

Dump the 3 ml liquid phase which was took out at the first time interval back to the reactor, mix with reagents perfectly, and, at the same time, take 3 ml out. The UV-absorbance at time= 15 sec. is 1.2745. Note that this is the reading corresponding to the mixture of 27 ml reagents continuously reacted with the active sites in the first 10 sec. and 3 ml of reagents in the circulation loop without contacting the active sites on the resin. So the real UV reading, in other words, the concentration of amino acid and anhydride can be solved as

$$1.2745 = \frac{27}{30} \times \text{Real Reading} + \frac{3}{30} \times 1.4524$$

Real reading = 1.255

Percent Error =(1.2745-1.255)/1.255 X 100%= 1.57%

At the third time interval (10-20 sec.)

The analysis is the same as in the second time interval

The UV-Absorbance at 25 sec. is = 1.148

$$1.148 = \frac{27}{30} \times \text{Real Reading} + \frac{3}{30} \times 1.186$$

Real reading = 1.143

Percent Error =(1.148-1.143)/1.143 x 100% = 0.44%

The error percentage, as expected, goes down as the percent reacted of the active sites increase.

As a summary, less than 2 percent of error was observed in this simple analysis about the circulation loop in the synthesis of polyphenylanaline at the number of amino acid residues on peptide n=3. A higher error will be resulted at faster reaction like lower number of residue or in the synthesis of polyserine. Graphically, a higher data point with respect to each data will be in the second order plot of $\ln((M-X_a)/M(1-X_a))$ versus time and will be lower in the shifting zero to first order plot.

APPENDIX C

EXPERTMENTAL RESULTS

The experimental results are presented in the Table XII in the percentage of active sites on the resin reacted as function of time, and $F1=X_A C_{AO}/Ln(1/(1-X_A))$, $F2=t/Ln(1/(1-X_A))$.

TABLE XII

EXPERIMENTAL RESULTS

Time(sec.)	Percent Reacted of Active sites	F1	F2
Peptide I (Polyserine, n=1)			
0	0.000	0	-
40	0.780	0.00515	26.41
80	0.885	0.00409	37.00
120	0.946	0.00323	41.03
160	0.961	0.00295	49.06
200	0.984	0.00235	47.88
n=3			
0	0.000	0	-
40	0.644	0.00623	38.70
80	0.826	0.00472	45.68
120	0.898	0.00393	52.53
160	0.939	0.00334	56.95
200	0.964	0.00289	60.09
240	0.979	0.00250	61.46
280	0.984	0.00237	67.48
n=5			
0	0.000	0	-
40	0.654	0.00615	37.60
80	0.782	0.00513	52.48
120	0.850	0.00447	63.17
240	0.943	0.00328	83.41
320	0.967	0.00282	93.46
400	0.983	0.00238	97.16
n=7			
0	0.000	0	-
40	0.554	0.00686	49.52
80	0.676	0.00599	70.86
120	0.747	0.00542	87.09
240	0.854	0.00443	124.41
320	0.894	0.00397	142.23
480	0.943	0.00329	167.53
600	0.965	0.00286	177.73

TABLE XII (CONTINUED)

Time(sec.)	Percent Reacted of Active Sites	F1	F2
n=9			
0	0.000	0	-
40	0.561	0.00681	48.58
120	0.705	0.00577	98.24
240	0.807	0.00489	145.52
360	0.863	0.00433	180.68
480	0.902	0.00387	206.10
600	0.928	0.00351	227.35
1080	0.976	0.00260	287.53
Peptide I (polyserine, reproduced, n=1)			
0	0.000	0	-
40	0.765	0.00528	27.62
80	0.868	0.00428	39.50
120	0.933	0.00345	44.39
160	0.950	0.00317	53.40
200	0.976	0.00261	53.62
n=3			
0	0.000	0	-
40	0.654	0.00616	37.68
80	0.827	0.00471	45.59
120	0.901	0.00389	51.88
240	0.984	0.00237	58.03
n=5			
0	0.000	0	-
40	0.669	0.00605	36.17
80	0.798	0.00498	50.01
120	0.877	0.00418	57.26
240	0.960	0.00298	74.56
280	0.978	0.00256	73.36
320	0.983	0.00241	78.53
n=7			
0	0.000	0	-
40	0.549	0.0068	50.23
80	0.677	0.0059	70.79
120	0.767	0.0052	82.37
180	0.835	0.0046	99.89
320	0.902	0.0038	137.76
480	0.955	0.0030	154.78
600	0.972	0.0027	167.80
n=9			

TABLE XII (CONTINUED)

Time(sec.)	Percent Reacted of Active Sites	F1	F2
0	0.000	0	-
40	0.490	0.00727	59.40
80	0.626	0.00636	81.34
120	0.680	0.00596	105.31
240	0.778	0.00516	159.46
360	0.836	0.00462	199.12
480	0.865	0.00431	239.70
600	0.897	0.00394	263.96
1080	0.934	0.00343	397.33
1320	0.956	0.00306	422.59
Peptide II (Polyphenylalanine, n=1)			
0	0.000	0	-
40	0.641	0.0201	29.38
80	0.767	0.0169	54.84
120	0.828	0.0151	68.13
180	0.887	0.0130	82.30
240	0.919	0.0117	95.10
360	0.948	0.0102	121.40
480	0.971	0.0088	135.09
n=3			
0	0.000	0	-
40	0.579	0.0215	46.17
80	0.700	0.0187	66.40
120	0.774	0.0167	80.45
180	0.845	0.0145	96.50
240	0.887	0.0130	109.77
360	0.934	0.0110	131.85
480	0.964	0.0092	143.54
n=5			
0	0.000	0	-
40	0.472	0.0237	62.53
80	0.575	0.0216	93.36
120	0.646	0.0200	115.41
240	0.767	0.0169	164.39
360	0.838	0.0148	197.33
480	0.886	0.0131	220.38
600	0.922	0.0116	234.96
840	0.958	0.0096	263.35

TABLE XII (CONTINUED)

Time(sec.)	Percent Reacted of Active Sites	F1	F2
n=7			
0	0.000	0	-
60	0.489	0.0234	89.19
120	0.596	0.0211	132.28
240	0.730	0.0179	183.03
360	0.803	0.0158	220.97
480	0.857	0.0141	240.08
600	0.897	0.0126	262.90
720	0.930	0.0112	270.68
1020	0.969	0.0089	293.59
n=9			
0	0.000	0	-
60	0.464	0.0239	96.10
120	0.554	0.0220	148.60
240	0.663	0.0196	220.52
360	0.726	0.0180	277.73
420	0.749	0.0174	303.46
Peptide II (Polyphenylalanine, reproduced, n=1)			
0	0.000	0	27.62
40	0.635	0.0202	39.68
80	0.756	0.0172	56.71
120	0.813	0.0156	71.57
180	0.877	0.0134	85.89
240	0.902	0.0125	103.32
360	0.933	0.0111	133.18
480	0.957	0.0097	152.54
n=3			
0	0.000	0	-
40	0.620	0.0206	41.34
80	0.739	0.0177	59.55
120	0.789	0.0163	77.12
240	0.878	0.0134	114.08
360	0.966	0.0091	106.46
480	0.981	0.0079	121.11
n=5			
0	0.000	0	-
40	0.485	0.0235	60.27
80	0.587	0.0213	90.46

TABLE XII (CONTINUED)

Time(sec.)	Percent Reacted of Active Sites	F1	F2
120	0.662	0.0196	110.62
240	0.780	0.0165	158.50
360	0.856	0.0142	185.76
600	0.940	0.0107	213.26
840	0.967	0.0091	246.24
n=7			
0	0.000	0	-
40	0.389	0.0254	81.19
80	0.562	0.0219	96.90
120	0.618	0.0206	124.69
240	0.743	0.0176	176.64
360	0.809	0.0157	217.45
480	0.863	0.0139	241.47
600	0.890	0.0129	271.82
720	0.928	0.0113	273.65
1020	0.962	0.0094	311.91
n=9			
0	0.000	0	-
60	0.490	0.0234	89.10
120	0.578	0.0215	139.09
240	0.688	0.0190	206.05
360	0.745	0.0175	263.44
420	0.772	0.0168	284.08
Peptide III (Polyserine, n=1)			
0	0.000	0	-
120	0.896	0.0039	52.99
240	0.959	0.0029	74.71
360	0.985	0.0023	84.73
n=3			
0	0.000	0	-
120	0.817	0.0048	70.65
240	0.925	0.0035	92.53
360	0.965	0.0028	107.19
480	0.985	0.0023	112.77
n=5			
0	0.000	0	-

TABLE XII (CONTINUED)

Time(sec.)	Percent Reacted of Active Sites	F1	F2
120	0.767	0.00526	82.24
240	0.870	0.00426	117.57
360	0.920	0.00363	141.98
480	0.946	0.00322	163.66
600	0.962	0.00293	182.97
720	0.971	0.00273	202.51
n=7			
0	0.000	0	-
120	0.659	0.00611	111.31
240	0.758	0.00533	168.93
360	0.823	0.00474	207.64
480	0.890	0.00402	216.64
640	0.907	0.00380	268.48
840	0.936	0.00339	304.94
1080	0.953	0.00311	352.61
n=9			
0	0.000	0	-
120	0.577	0.00670	139.11
240	0.689	0.00589	205.14
360	0.751	0.00539	258.22
480	0.801	0.00495	297.03
600	0.833	0.00464	334.38
720	0.854	0.00443	373.46
840	0.877	0.00417	399.41
1080	0.909	0.00379	450.39
1320	0.924	0.00357	510.45
Peptide IV (Polyphenylalanine, n=1)			
0	0.000	0	-
60	0.704	0.01860	49.22
120	0.814	0.01556	71.24
180	0.884	0.01320	83.45
240	0.922	0.01163	94.02
360	0.964	0.00930	107.85
480	0.983	0.00766	116.14
n=3			
0	0.000	0	-
60	0.591	0.02128	67.09
180	0.793	0.01618	113.99

TABLE XII (CONTINUED)

Time(sec.)	Percent Reacted of Active Sites	F1	F2
240	0.840	0.0147	130.61
360	0.901	0.0125	155.33
420	0.920	0.0116	165.49
480	0.935	0.0109	174.76
600	0.962	0.0094	197.99
720	0.973	0.0086	195.93
n=5			
0	0.000	0	-
120	0.497	0.0232	174.27
240	0.613	0.0207	252.44
360	0.691	0.0189	306.10
480	0.740	0.0176	355.48
600	0.784	0.0164	391.04
840	0.842	0.0146	453.85
1080	0.886	0.0131	496.39
1560	0.941	0.0106	549.49
1800	0.959	0.0096	559.79
n=7			
0	0.000	0	-
120	0.430	0.0246	213.11
240	0.548	0.0222	301.57
360	0.636	0.0202	356.17
480	0.681	0.0191	418.98
840	0.801	0.0159	519.68
1320	0.882	0.0132	615.47
1560	0.909	0.0121	649.94
2400	0.965	0.0092	712.49
n=9			
0	0.000	0	-
120	0.426	0.0247	215.87
240	0.540	0.0223	308.86
480	0.667	0.0195	435.54
720	0.743	0.0175	528.89
960	0.801	0.0159	594.35
1380	0.852	0.0143	720.76
1740	0.879	0.0133	822.75
1980	0.896	0.0127	872.75
2340	0.913	0.0120	954.71
3300	0.937	0.0108	1187.25

TABLE XII (CONTINUED)

Time(sec.)	Percent Reacted of Active Sites	F1	F2
Peptide V (Polyphenylalanine, n=1)			
0	0.000	0	-
120	0.870	0.0137	58.79
240	0.941	0.0106	84.50
360	0.969	0.0089	103.16
n=3			
0	0.000	0	-
120	0.811	0.0156	71.87
240	0.899	0.0125	104.34
360	0.939	0.0107	128.07
480	0.961	0.0094	147.20
600	0.975	0.0084	161.60
n=5			
0	0.000	0	-
120	0.687	0.0190	103.21
240	0.782	0.0165	157.30
360	0.831	0.0150	202.19
480	0.868	0.0137	236.93
600	0.892	0.0128	268.94
720	0.930	0.0112	270.56
1080	0.956	0.0098	344.92
n=7			
0	0.000	0	-
120	0.431	0.0246	212.52
240	0.524	0.0227	323.28
360	0.583	0.0214	411.12
600	0.660	0.0196	555.77
720	0.684	0.0191	623.76
1080	0.732	0.0178	818.44
1320	0.751	0.0173	949.18
n=9			
0	0.000	0	-
120	0.393	0.0253	240.36
240	0.480	0.0236	366.63
360	0.535	0.0225	471.96
480	0.571	0.0217	566.89
1320	0.675	0.0193	1171.29

APPENDIX D

SAMPLE CALCULATIONS OF RATE CONSTANTS FOR IDEAL SECOND ORDER WITH ACTIVATION ENERGY AND PROPOSED REACTION RATE EXPRESSION

SAMPLE CALCULATION OF RATE CONSTANTS FOR IDEAL SECOND ORDER RATE EQUATION WITH ACTIVATION ENERGY

Consider the reaction

$$A + X \longrightarrow \text{Products}$$

with corresponding rate equation

$$-\gamma_A = -\frac{dC_A}{dt} = -\frac{dC_X}{dt} = k_2 C_A C_X$$

$$-\gamma_A = C_{A0}\frac{dC_A}{dt} = k_2(C_{A0} - C_{A0}X_A)(C_{X0} - C_{A0}X_A)$$

Letting $M=C_{X0}/C_{A0}$ be the initial molar ratio of reactants,

$$-\gamma_A = C_{A0}\frac{dC_A}{dt} = k_2 C_{A0}^2(1-X_A)(M-X_A)$$

which on separation and formal integration becomes

$$\int_0^{X_A} \frac{dX_A}{(1-X_A)(M-X_A)} = C_{A0}k_2\int_0^t dt$$

After breakdowm into partial fractions, integration, and rearrangement, the final result in the following form

$$\ln \frac{M-X_A}{M(1-X_A)} = (C_{X0} - C_{A0})\, k_2 t$$

So, the plot of $\ln((M-X_A)/M(1-X_A))$ versus t(Time) will give the slope equal to $(C_{X0}-C_{A0})\, k_2$

Example: for n=3 at the synthesis of Peptide II(Phe)
at 50% conversion of active sites of resin, Figure 13 shows that the slope = 0.00785 and the $(C_{X0}-C_{A0})$ = 0.0161

$k_2 = 4.876 \times 10^{-1}$

Similarly, for synthesis of Peptide IV(Phe), $k_2 = 3.97 \times 10^{-1}$

According to the Arrhenius's law, the activation energy can be calculated as 2.917 Kcal/gmole.

SAMPLE CALCULATION OF RATE CONSTANTS FOR PROPOSED RATE EQUATION:

$$-\gamma_A = -\frac{dC_A}{dt}\left[\frac{\text{mole/l}}{\text{sec}}\right] = \frac{K_1 C_A}{K_2 + C_A}$$

where C_A is the amino terminus concentration mmol/ml

Example: Let $F1 = X_A C_{A0} / \ln (1/(1-X_A))$

$F2 = t/ \ln (1/(1-X_A))$

For the synthesis of Peptide II(Phe)

Resin weight = 1.38 gram (2% DVB Cross-linking)

Reaction volume = 30 ml DCM

Loading of resin = 0.7 mmol/ g resin

Reaction temperature 26 C, Mixing rate = 200 RPM

Mole ration =1.5

$C_{A0} = 3.22 \times 10^{-2}$ mole/l DCM

$K_1 = (0.0215-0.0145)/(46.17-96.50) \times 10^2 = -1.39 \times 10^{-2}$

K_2 intercept $= 2.78 \times 10^{-2}$

$K_1 \equiv [\text{mole/l sec}]$

$K_2 \equiv [\text{mole/l}]$

VITA

Wen-Yih Chen

Candidate for the Degree of

Doctor of Philosophy

Thesis. A KINETIC STUDY OF SOLID PHASE PEPTIDE SYNTHESIS FOR THE PRODUCTION OF POLYPHENYLALANINE AND POLYSERINE WITH LOW EXCESS OF THE SYMMETRICAL ANHYDRIDE

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